UNIVERSITY OF BIRMINGHAM

The Role of Thyroid Hormone Transporters Monocarboxylate Transporter 10 (MCT10) and Monocarboxylate Transporter 8 (MCT8) in Extravillous Trophoblasts.

MPhil Thesis of Juhela Choudhury

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Abstract

Thyroid hormones (TH) are important for fetal and placental development. Monocarboxylate transporters 8 and 10 (MCT8, MCT10) are effective plasma membrane TH transporters expressed in the human placenta from 6 weeks of gestation. Both have been localized to human villous trophoblasts and extravillous trophoblasts (EVTs).

Aims: Using HTR-8/SVneo cells as a model of 1st trimester EVTs, we assessed 1) Triiodothyronine (T3) effects on gene expression and cell proliferation; 2) the effect of altered MCT8 or MCT10 expression on proliferation and apoptosis; and whether effects were T3-mediated.

Methods: 1) Cell proliferation was assessed by the MTT assay at 24-96hrs. Gene expression was assessed by quantitative QPCR (2-48h). **2)** MCT8 was down-regulated using siRNA, and MCT8 and MCT10 were over-expressed by plasmid transfection. Effects of altering expression were assessed by MTT assay (Proliferation) and Caspase3/7 activity (Apoptosis).

Results: We observed that T3 treatment did not affect HTR-8/SVneo proliferation nor alter the mRNA expressions of TH responsive gene, Connexin43 (Cx43) and the pre-receptor regulators of T3 action, deiodinases 2 and 3 (D2, D3), nor its own transporters, MCT8 and MCT10. However, T3 treatment was observed to decrease the expression of TH receptor β1 (TRβ1) (ANOVA $p<0.05$). Over-expression of MCT8 or MCT10 reduced cell proliferation by 36% and 29% respectively (ANOVA $p<0.05$) in the presence of T3. Apoptosis was reduced, independently of T3 treatment, by 24 and 31% (+/- T3 respectively) following overexpression of MCT8, and by 12 and 19% (+/- T3 respectively) following overexpression of MCT10, respectively (ANOVA $p<0.005$). Conversely, down-regulation of MCT8 resulted in increased apoptosis independently of T3 ($p<0.003$).

Conclusions: HTR-8/SVneo cell proliferation does not change with T3 treatment. In addition, MCT8 and MCT10 may have both T3-dependent (suppress proliferation) and T3-independent (apoptosis) effects in EVTs. This suggests an important role for MCT8 and MCT10 in human placental development.

18S rRNA	**18 S** ribosomal RNA
3,3’-T2	3,3'-Diiodothyronine (3,3'-T2)
4F2Hc	obligate hetromer of LAT1
Ab	antibody
AMV	Avian Myeloblastosis Virus
ATF-2	activation transcription factor 2
BeWo	human placenta choriocarcinoma **cell** line.
cdks	cyclin-dependent-kinases
cDNA	complementary DNA
CNS	central nervous system
COS1 cells	cell line derived from kidney **cells** of the African green monkey
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding proteins
CRYM	μ-crystallin protein
CT	cytotrophoblasts
cx43	Connexin43
D1/ D2/ D3	deiodinases
DMEM	Dulbecco’s modified Eagle's medium
DMSO	dimethylsulf-oxide
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol or Cleland's reagent
E2F	family of transcription factors
ECL	Enhanced Chemiluminescence
EGF	epidermal growth factor
EVT	extravillous trophoblasts
FACS	fluorescence activated cell sorting
FBS	Fetal Bovine Serum
HA	haemagglutinin tag

HCG	human chorionic gonadotropin
HIF-1	hypoxia inducible factor-1
hMCT8/ *hMCT10*	human MCT8/MCT10
IUGR	intrauterine growth restriction
JAR	human placenta choriocarcinoma cells
JEG-3	choriocarcinoma extravillous trophoblast like cell line
Ki67	prototypic cell cycle related nuclear protein
LDH	lactate dehydrogenase
LAT1	L-type Amino Acid Transporter 1
LAT2	L-type Amino Acid Transporter 2
MCT (1 – 14)	Monocarboxylate Transporters
Mdm2	murine double minute 2
MTT	**methyl** thiazol **tetrazolium**
N2a-β	neuroblastoma cells
NaOH	sodium hydroxide
NK	natural killer cells
NOX1	NADPH oxidase 1
NTCP	**Na/aurocholate cotransporting polypeptide**
OATP/ OATP4A1/OATP1A2	organic anion transporting polypeptides
PBS	phosphate buffered saline
pcDNA3.1+	plasmid
PCR	Polymerase Chain reaction
PE	preeclampsia
PEST	peptide sequence rich in praline, glutamic acid, serine and threonine
PVDF	poly-vinylidene fluoride
Q-PCR	real time polymerase chain reaction
rT3	reverse 3, 3, 5-tri-iodothyronine
RASM	rat aorta smooth muscle

Rb	retinoblastoma protein
RNAi	RNA inhibition
RPMI 1640	media developed by Roswell Park Memorial Institute for cell culture
RT	reverse transcription
RT-PCR	reverse transcription PCR
RXR	**retinoid X receptor**
scRNA	scramble ribonucleic acid
SDS	sodium dodecylsulphate
SGH-PL4	extravillous trophoblast derived cell line
siRNA	small interfering RNA
ST	syncytiotrophoblast
T3	3, 3, 5-tri-iodothyronine
T4	3, 5, 3', 5'-tetraiodothyronine (or thyroxine)
TAT1	T-type amino acid transporter 1
TBST	Tris-Buffered Saline Tween-20 (),
TGF-β	transforming growth factor-β
TH	thyroid hormone
TR	thyroid hormone nuclear receptors
TRα/ TRα1/ TRα2	thyroid hormone receptor alpha
TRβ/ TRβ1/ TRβ2	thyroid hormone nuclear receptor beta
TRE	thyroid hormone responsive element
TSH	thyroid stimulating hormone
VO	vector only
WT	wild type
XPCT	X-linked PEST containing transporter

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1. Introduction

Thyroid hormones (TH) are iodinated tyrosine derivatives produced and secreted by the thyroid gland. The detection of significant levels of T4 and T3 in embryonic tissues, (Calvo *et al* 1990; Calvo RM *et al* 2002) and in neonates with congenital hypothyroidism (Vulsma *et al* 1989) demonstrates that the transplacental delivery of maternal THs to the fetus occurs in humans. TH transfer is thought to be particularly important in early pregnancy, before the onset of endogenous fetal TH production; which does not usually occur till the 14th week of gestation (Vulsma *et al* 1989; Calvo *et al* 2002; Morreale de Escobar *et al* 2004; Terán-Pérez *et al* 2009).

We and others have contributed to the evidence supporting the notion that the development of the fetal central nervous system (CNS) is thyroid hormone responsive from the first trimester and throughout pregnancy; and that TH is important also in neonatal brain development (Chan *et al* 2003; Büyükgebiz 2006; Morreale de Escobar *et al* 2007; Terán-Pérez *et al* 2009). Even minor perturbations in maternal TH status, particularly during the first trimester, were shown to be associated with neurodevelopmental abnormalities and increased risk of miscarriage and preterm labour (Kilby *et al* 1998; Chan *et al* 2006). Overt hypothyroidism and hyperthyroidism in pregnant women for example, is also known to be associated with many adverse obstetric outcomes including spontaneous abortion, stillbirths, placental abruptions, fetal growth restriction and pregnancy-induced hypertension as well as

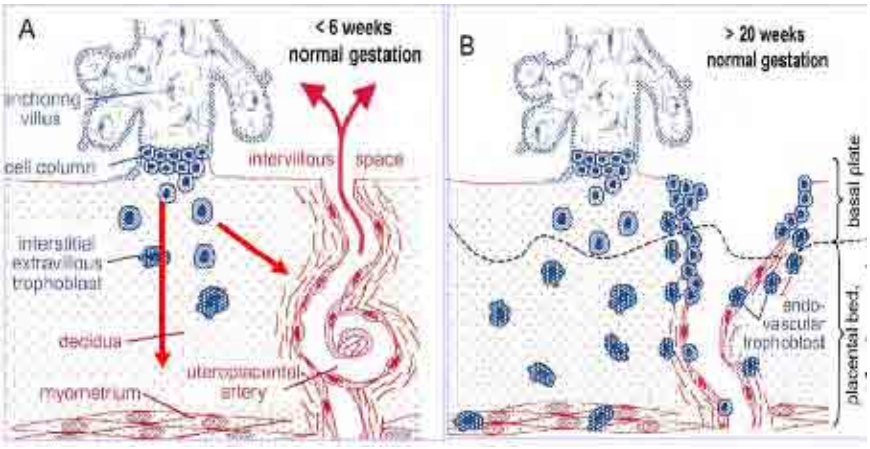
neurological deficits in children (Morreale de Escobar *et al* 2007). This suggests that THs may also play a critical role in modulating the development of the placenta during pregnancy in addition to the postulated role of TH in fetal CNS development.

The Human Placenta

As a complex organ with specialized metabolic, hormonal and immunological functions, the placenta is the means by which the nutritive, respiratory and excretory needs of the fetus are carried out. It serves as the interface between the maternal uterine tissues and the conceptus. An important anatomic unit of the placenta are the villi, the formation of which begins immediately after implantation of the blastocyst to the uterine wall. Each villus consists of an outer layer of trophoblasts and an inner mass formed of the somatic mesoderm (Wooding *et al* 1994). The trophoblast shell of the villi is comprised of an internal layer of mononucleated cytotrophoblasts (CT). CTs go on to form an external layer of specialised multinucleated villous syncytiotrophoblasts (ST), involved in fetal-maternal exchange and the secretion of placental hormones into maternal blood (Adé-Damilano *et al*, 2003).

At the base of anchoring villi, CTs break through the villous layer giving rise to extravillous trophoblasts (EVT), which are a population of proliferative and migratory placental cells that invade the uterine stroma from trophoblastic cell columns (**Figure 1**), (Kaufman *et al*

2003). They comprise a major part of the invasive component of placentation.



(a) **(b)**

Figure 1. Anchoring villi. (a) Extravillous trophoblasts (EVT) migrate into the uterine stroma from trophoblast cell columns, invading the decidua and transforming maternal spiral arteries to high capacitance vessels, for adequate perfusion of the placental bed. **(b)** Remodelling of spiral arteries and invasion of the myometrium following EVT invasion at 20 weeks of normal gestation (Kaufman *et al* 2003).

EVTs switch from the proliferative to the invasive pathway as they reach the decidua, at around 10-12 weeks of gestation (Pijnenborg *et al* 1983). Interstitial EVTs invade the maternal uterine decidua, while endovascular EVTs transform maternal spiral arteries into low resistance, high capacitance vessels; to meet the level of required utero-placental perfusion, i.e. an adequate blood flow to the placental bed and the developing fetus (Kaufman *et al* 2003).

Decidual immune cells, such as macrophages and NK cells facilitate deep invasion of EVT cells up to myometrial portions of spiral arteries. Adequate invasion has to be balanced against the risk of

uncontrolled and damaging invasion of maternal tissues. Thus, the development of the human placenta in the first half of gestation has to be a tightly regulated process of proliferation, differentiation, invasion by trophoblasts at the deciduas basalis and spiral arteries, and apoptosis. The role of apoptosis is believed to be important to ensure that trophoblast turnover occurs as normal without interference by the maternal inflammatory response (Laoag-Fernandez *et al* 2004).

EVTs are highly differentiated uninuclear cells that have two distinct phenotypes, they are proliferative and invasive. They also undergo apoptosis, an activity of EVTs that appears to be more significant than their proliferative potential (Kaufmann *et al* 1997). Both the increased EVT differentiation (to placental bed giant cells which have lost their ability to migrate and invade) and apoptosis of invasive EVTs, represents a protection mechanism that prevents deeper penetration of trophoblasts into the uterine wall (Kadyrov *et al* 2003; Von Rango *et al* 2003; Huppertz *et al* 2006; Lunghi *et al* 2007). The latter stages (effecter stage) of EVT apoptosis in particular, has been demonstrated to be detectable in the lower part of the endometrium (or myometrium), where prevention of further invasion of EVTs deep into the placental bed would be required (Kadyrov *et al* 2003; Von Rango *et al* 2003).

In the case of inadequate EVT invasion and incomplete remodelling of spiral arteries, placental insufficiency (where there is poor

placental perfusion) may impede fetal growth. This has been highlighted as a feature in malplacentation syndromes such as Intrauterine Growth Restriction (IUGR), Preeclampsia (PE) and late miscarriage; with the associated risk of iatrogenic preterm delivery and increased perinatal mortality and morbidity (Brosens *et al* 1977; Kim *et al* 2002). Thus, adequate invasion is a particularly important milestone in feto-placental development.

There is growing evidence to support the importance of a number of hormonal and growth factors such as Epidermal Growth Factor (EGF), and cytokines in the regulation of trophoblast growth and activity (such as invasion), which plays a major role in determining placental development (Lash *et al* 1999; Barber *et al* 2005). T3 is one such factor that is believed to play an important role, in the regulation of trophoblast activity, as well as fetal development. Therefore, it is important to ensure that requisite amounts of TH reach TH responsive cells in the fetus and placenta at each gestational stage.

Regulation of TH Action

T4 (3, 5, 3', 5'-tetraiodothyronine, or thyroxine) is the predominant TH that is secreted by the thyroid gland, although T3 (3, 3, 5-tri-iodothyronine), is the active TH ligand which elicits its actions via binding to TH nuclear receptors (TRs) in TH responsive cells (Yen *et al* 2001). TRs, in conjunction with RXRs, are nuclear transcription factors which regulate the expression of TH responsive genes. TR

isoforms are produced by alternative splicing of the *TRα* and *TRβ* genes, and are differentially expressed in most tissues. The main T3-binding isoforms are *TRα1* and *TRβ1. TRα2* does not bind T3 and is thought to regulate the activities of the other isoforms. This isoforms is only observed in the pituitary.

THs may act through non-genomic mechanisms, although it is believed that the biological action of T3 is principally dependent on the interaction of T3 with TRs; which in turn, is controlled by a number of factors (**Figure 1. 2**) including;

i) The physiological concentrations of T4 and T3 in the circulation.
ii) T4 and T3 metabolism, which occurs in part by the action of Deiodinases (D1, D2 and D3); regulating TH metabolism via the outer/inner ring deiodination of iodothyronines (Köhrle 1999).
iii) The differential expression of TH receptor (TR) isoforms; nuclear transcription factors involved in the regulation of T3 responsive genes (Kilby *et al* 2005).
iv) Type of TR heterodimerisation partners (RXRs) as well as the TRE structure in the promoters of target genes.
v) The cellular availability of co-activators and co-repressors (Edwards 2000).
vi) The transport of TH in and out of cells via plasma membrane transporters.

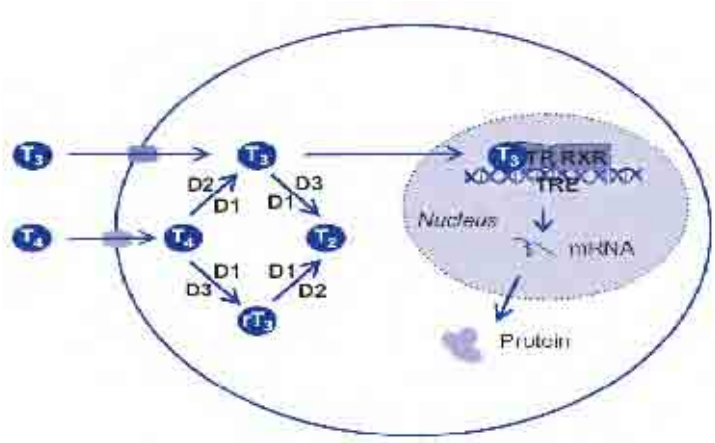


Figure 1. 2. The intracellular activity of TH is determined by a number of factors: firstly, the availability of nuclear TH receptors (TRs) and other receptors such as the nuclear retinoid X receptor (RXR) (RXRs and TRs form heterodimers); secondly, the action of Deiodinases (D1, D2 and D3) responsible for the conversion of THs between active and inactive forms; and finally the action of TH transporters, which modulate the uptake and efflux of TH in and out of cells (**Jansen *et al* 2005**).

Even though the circulating concentration of T4 is the primary factor in determining TH availability to tissues, all of the above factors are potential areas for regulation of TH action in the placenta.

Thus, TH action may be modulated at the pre-receptor (e.g. TH levels, availability), or the receptor level (TR isoforms, interacting partners). In addition, binding to the T3 responsive element (TRE) in the 5'-upstream promoter region of TH responsive genes can result in either the stimulation or repression of gene expression (Zhang *et al* 2000). 'Positive' TREs are those that are present in T3 activated promoters, while 'negative' TREs are present in T3 inhibited promoters.

Differential Expression of Deiodinases and TRs in Placenta

Our group and others have previously demonstrated the widespread expression of TRs and D2 and D3 in utero-placental tissues from normal pregnancies throughout gestation, (Salvatore *et al* 1995; Koopdonk-kool *et al* 1996; Kilby *et al* 1998; Chan *et al* 2003; Barber *et al* 2005). These findings are in line with the hypothesis that placental development is TH responsive form early gestation.

Only D2 and D3 are expressed in the placenta. They are integral membrane proteins, localised predominantly in the endoplasmic reticulum (where D2 is preferentially expressed) and the plasma membrane (where D3 is preferentially expressed). D3 (responsible for inactivating T3 and T4, **Figure 1. 2**) is the predominant deiodinase expressed in villous STs, as well as in CT layers of the placenta at each stage of gestation. STs form the primary (initial) barrier to the transfer of TH from mother to the fetus therefore, increased expression of D3 at this locality is suggestive of a protective role against excess TH levels in the fetus (Chan *et al* 2003). Moreover, the expression of D2 (converts T4 to T3, **Figure 1. 2**) in first trimester villous trophoblasts, primarily in CTs; is indicative of the potential importance of T3 in CT development (Chan *et al* 2003).

Trophoblast Regulation and Placental Development

The differentiation of CTs to STs and EVTs is a significant part of placentation, as is trophoblast motility, proliferation and invasion. In

addition to potential regulation by T3, trophoblast functions are believed to be regulated both spatially and temporally, by a number of local growth factors, hormones, transcription factors, specific genes, cytokines, oxygen levels, growth factor binding proteins and proteoglycans, as well as components of the extracellular matrix. For example, reduced oxygen levels stimulate CT proliferation and inhibit EVT and ST differentiation, by inducing the hypoxia inducible factor-1 (HIF-1) (Lala *et al* 1998).

The possible role of T3 in placentation has been previously investigated. In-vitro studies using primary EVTs show that T3 increased cell proliferation (Maruo T *et al* 1991, 1994) and invasion, in association with the increased activation of matrix metalloproteinase's (Oki *et al* 2004). Furthermore, Maruo *et al* (1991) proposed that T3 was also a promoter of endocrine function in early placenta, by stimulation of the secretion of human chorionic gonadotropin (HCG), oestradiol and progesterone. In previous work, our group had also observed increased motility and invasion, in an EVT derived cell line, SGH-PL4 cells, in response to T3 (Barber *et al* 2005). Other studies have reported a potential role of T3 in the suppression of apoptosis in first trimester trophoblasts, and in promoting the differentiation of villous trophoblasts to EVTs (Maruo *et al* 1991; Matsuo *et al* 1993; Laoag-Fernandez *et al* 2004; Huppertz *et al* 2006).

Moreover, in addition to the observed stimulatory effects of T3 on trophoblast function, T3 was also shown to increase Epidermal Growth Factor (EGF) production by CTs (Maruo *et al* 1991; Matsuo *et al* 1993). EGF is a potent mitogen that has been shown to act as an autocrine factor in regulating early placental growth and function (Maruo *et al* 1987-1997). The potential synergistic effect of both EGF and T3 on trophoblast activity had been investigated previously by our group and others. We reported on: increased motility of an EVT derived cell line SGH-PL4 in co-incubation experiments of EGF and T3 (EGF at 50 mg/ml and T3 at 1 and 10 nm) where presence of T3 was found to enhance EGF-stimulated cell migration by up to around 25%; increased survival of first trimester CT cells, and increased invasion of SGH-PL4 cells by up to two fold (Barber *et al* 2005).

T3 promotion of EGF production in first trimester CTs, has also been reported to increase D2 activity (Canettieri *et al* 2008), and therefore T3 availability and subsequent T3 action in these cells (Matsuo *et al*, 1993). Thus, EGF could facilitate the positive self-potentiation of T3 effects in CTs. A more recent study by Moll *et al* (2007) reported EGF to also reduce apoptosis of trophoblasts.

Thus, the biological activity of T3 in trophoblasts is in part determined and regulated by factors that control of the intracellular T3 pool, i.e. deiodinase activity and the transport of TH into

trophoblasts. The transport of TH into trophoblasts in particular has been an area of interest and potential investigation for our group.

In developmental disorders such as IUGR, the normal biology of THs appears to be disrupted. Upon measuring concentrations of free T4 and T3 in the serum of IUGR and normal fetuses, we and others have previously reported marked reductions in circulating levels of maternal T4 and T3 in severely growth restricted human fetuses. In addition, in the IUGR placenta sample, we had previously reported an up-regulation of TRs, compared to normal gestational age matched controls, reflecting a possible placental compensation for the low circulating levels of maternal TH (Kilby *et al* 1998). In this way, differential expressions of TRs and TH transporters have been observed by our group and others in previous studies investigating TH impact in tissue development.

Transport of TH in the Placenta

It has been postulated that impaired TH transport into CNS cells have resulted in significantly impaired neurodevelopment (Heuer *et al* 2007). MCT8 is a highly effective TH transporter and to date has not been found to transport any other compound.

The recognition of membrane transporters as potentially important regulators of TH activity comes with the identification of MCT8 gene mutations, in individuals with profound neurodevelopmental defects, although the causative link between T3 activity and such

neuroimpairment has not yet been established (Dumitrescu *et al* 2004; Friesema *et al* 2004). Several other different TH transporters have also been identified and categorised according to substrate specificities and their mechanisms of transport. To date, there are six different TH transporters known to be present in the placenta: MCT8, MCT10, OATP1A2, OATP4A1, LAT1 and LAT2 (Chan *et al* 2009). These transporters can be categorised as:

i) The **Organic Anion Transporting Polypeptides** (**OATP**), with several members recognised as TH transporters including OATP4A1 and OATP1A2.

ii) **Amino Acid Transporters** such as the L-type Amino Acid Transporter (LAT) which is involved in the transport of both TH and amino acids.

iii) **Monocarboxylate Transporters (MCT)**. Despite there being 14 MCTs recognised to date - only two have so far been identified to be TH transporters: MCT8 and MCT10.

This study focuses on the roles of MCT8 and MCT10 in EVTs. MCT8 and MCT10 do not transport monocarboxylates in a proton dependent manner as has been shown for MCTs 1 - 4, although they share a similar structure and homology to MCTs. However, MCT8 and MCT10 do effectively transport TH (Friesema *et al* 2003, 2006, 2008). Furthermore, MCT10 has been shown to transport aromatic amino acids. MCTs 7, 9, 11 and 14 remain to be characterised.

MCT8

Human *MCT8* was first cloned in 1994, the gene being located on chromosome Xq13.2. Originally named X-linked PEST containing transporter (or XPCT) MCT8 has two translational start sites giving rise to two possible protein versions, and an intracellular PEST domain at the N-terminus, which encodes for a 539 or 613 amino acid protein (depending on the translational start site) with 12 putative trans-membrane domains (Friesema *et al* 2004). Its function remained elusive until 2003 when it became identified as a potent transporter of TH, with a preference for T3 transport in humans (Friesema *et al* 2003, 2006). Its biological importance however, became known after the identification of loss-of-function MCT8 mutations in males affected with severe neurodevelopmental disorder, from un-related families. The affected males presented with severe psychomotor retardation, hallmarked by hypotonia of the axial muscles, spastic or dystonic quadriplegia, and severe cognitive impairment. The majority of patients were not able to sit, stand, or walk without support, and development of speech was absent. Another hallmark of the condition was abnormal thyroid function tests, particularly elevated serum T3, borderline low T4 and normal-high thyroid stimulating hormone (TSH). The first identification of this neurological phenotype was published in 1944, also known as Allan-Herndon-Dudley syndrome, after the authors (Allan *et al* 1944).

MCT8 was identified as a potent transporter of TH in 2003 (Friesema *et al* 2003). It was found to transport T4, T3, rT3, and 3,3'-T2 from studies in Xenopus oocytes expressing exogenous rat MCT8. Expression of rat MCT8 in these cells was demonstrated to induce an increase up to ten fold in T3 and T4 uptake with a slight preference in the transport of T3 relative to T4, as indicated by the K_m values; which were 4.0μM, and 4.7μM respectively. The rate of transport of T4 and T3 was observed to be much greater by MCT8 than by other TH transporters such as NTCP and members of the OATP family. In addition, TH transport by MCT8 was not found to be proton or sodium dependent, although there appeared to be a mild dependency on sodium for T4 transport (Friesema *et al* 2003).

Friesema *et al* subsequently reported on the functional characterisation of human MCT8 (hMCT8) as a plasma membrane transporter of iodothyronines, in transiently transfected COS1 cells and JEG3 cells by analysis of iodothyronine uptake and metabolism. Transfection of hMCT8 into these cells produced a 2 to 3 fold increase in the uptake of T3 and T4. Increased MCT8 expression in these cells also produced elevated metabolism of T4 upon cotransfection with D2 and D3, and elevated metabolism of T3 on contransfection with D3. Affinity labelling of hMCT8 was demonstrated upon incubating hMCT8 transfected cells with *N*-bromoacetyl-[125I] T3 (Friesema *et al* 2006). Cotransfection studies using cytosolic thyroid hormone-binding protein μ-crystallin (CRYM) indicated that hMCT8 facilitated both cellular uptake and efflux of

T4 and in particular T3 across the plasma membrane (Friesema *et al* 2008).

So far MCT8 has not been found to transport other substrates including (aromatic or neutral branch chain) amino acids, iodothyronine sulphates and sulphamates, lactate or other monocarboxylates (Friesema *et al* 2003; Visser *et al* 2007).

MCT8 is expressed widely in many tissues including in liver, kidney, skeletal muscle, heart, brain as well as in the thyroid. Our group is presently investigating the potential importance of MCT8 in mediating T3 responsiveness in placenta and in transplacental TH transport. We have demonstrated the presence of MCT8 in first trimester villous CTs, as well as in endovascular EVTs. In addition, we have demonstrated the pattern of MCT8 expression in both villous and EVTs to be similar with that previously described for TRs and D2, in the first trimester. The co-localisation of the full complement of TH apparatus to mediate T3 action is consistent with the notion that trophoblasts are T3 responsive from early gestation (Matsuo *et al* 1997; James *et al* 2007).

MCT8 mutations have been associated with X-linked severe psychomotor retardation and elevated serum T3 levels in affected male patients, where some male patients present with a syndrome known as Allan-Herndon-Dudley syndrome (Dumitrescu *et al* 2004; Friesema *et al* 2004). Every mutation in each family (over 20 have

been found so far) is different and located in different parts of the gene. There have also been polymorphic variants identified in MCT8, (in which there is a change of serine to proline at position 107) which are not associated with a neurological phenotype, nor with altered serum TH levels, MCT8 mRNA expression, or the expression of TH responsive genes (Lago–Leston *et al* 2009).

MCT10

Human *hMCT10* (*Slc16a10*) is located on chromosome 6q21-q22. It has a single translational site, which encodes for a 515 amino acid protein and like MCT8 has 12 putative trans-membrane domains. MCT10 shares a 49% amino acid homology with MCT8 (Kim *et al* 2001; Friesema *et al* 2008). It is the only other MCT that has a PEST domain at its N terminal as MCT8 does (Rechsteiner *et al* 1996).

In previous work, Zhou *et al* (1990, 1992) described a T-type amino acid transporter, possibly involved in iodothyronine and amino acid uptake. In 2001, Kim *et al* described the expression, cloning and characterization of such a transporter in humans and rats. Initially identified as a basolateral aromatic amino acid transporter, the T-type amino acid transporter 1 (TAT1) was categorised as a sodium dependent low affinity transporter of amino acids with substrate specificity for anions (Kim *et al* 2001). Also exhibiting sequence similarity to proton coupled MCTs, TAT1 was further investigated for proton coupled transport, by Kim *et al* (2001). They were able to conclude that TAT1 was more akin to the MCT family as suggested

by its substrate recognition and strong amino acid sequence similarity to MCT8. However, in their 2001 paper Kim *et al* concluded that TAT1, or MCT10 as it was renamed, was not a transporter of T3 and T4.

Friesema *et al* retested the activity of MCT10, and reported that MCT10 was an effective transporter of THs in COS1 cells (Friesema *et al* 2006). They also demonstrated MCT10 to be involved in both the influx and efflux of TH, with a greater preference for T3 uptake, relative to T4, as demonstrated for MCT8 previously. Furthermore, the uptake of T3 was observed to be greater by hMCT10 than it was for hMCT8, although hMCT10 was less active toward T4 relative to hMCT8 (Friesema *et al* 2003 – 2008; Visser *et al* 2007).

It has been postulated that MCT8 and MCT10 may have important functions other than TH transport (James *et al* 2007). Unlike MCT10, however, MCT8 has not been found to transport other substrates such as aromatic amino acids (Friesema *et al* 2006). MCT10 expression was demonstrated in human and rodent tissues, i.e. the liver, kidney, skeletal muscle, intestine, heart and placenta.

Ramadan *et al* (2006) investigated the functional role of MCT10 in mouse, and reported that MCT10 was a basolateral epithelial transporter involved in the net efflux pathway for aromatic amino acids in the renal proximal tubules, small intestine and peri-venous hepatocytes. They localised MCT10 to the basolateral membrane of

intestinal villi cells and the proximal tubules. Our group demonstrated the expression of MCT10 protein and mRNA in human placenta from as early as 6 weeks of gestation, where MCT10, like MCT8 was found to be localised in both villous trophoblasts and EVTs (Loubière *et al* 2010).

Unlike for MCT8, no MCT10 mutations have been identified yet, although a single non-synonymous polymorphism (a Lysine to Glutamine change) was identified by Van der Deure *et al* (2007), though this was not found to impact on phenotype.

The transporting activity of MCT8 and MCT10 at the plasma membrane may depend on a number of factors. Their relative affinities for the different THs (as indicated by their K_m values), the gradients that exist across the individual plasma membranes for each TH metabolite, as well as the presence of other compounds transported by these proteins, which may act as competitive inhibitors (Chan *et al* 2009).

MCT8 and MCT10 Expression in Human Trophoblast

The exact role of MCT10 and MCT8 in trophoblasts is unknown. Both are expressed increasingly in the human villous placenta with advancing gestation. Expression from 6 weeks suggests a role at every stage of development. The expression of MCT8 in placenta from early gestation is therefore, compatible with an important role during fetal development and a specific role in placental

development. Studies by our group had shown MCT10 and MCT8 expression to increase with advancing gestation in human placenta via quantitative RT-PCR (Chan *et al* 2006).

IUGR is a developmental disorder associated with malplacentation and restricted fetal development. In utero sampling of cord blood (Cordocentesis) has showed a marked reduction in the fetal circulating free T4 and free T3 levels in growth restricted human fetuses relative to that observed for normal age-matched controls (Kilby *et al* 1998). In placenta obtained from pregnancies complicated by severe IUGR requiring preterm delivery (25-32 weeks), we observed *MCT10* mRNA expression to be significantly reduced compared to age matched appropriately grown for gestational age controls (Loubière *et al* 2010).

In contrast, MCT8 protein was more greatly expressed in placenta obtained from pregnancies complicated by severe IUGR requiring preterm delivery (25-32 weeks) (Chan *et al* 2006; Loubière *et al* 2009). These alterations in MCT10 and MCT8 expressions in IUGR placenta may reflect a compensatory response to the pathology, or a part of the pathogenesis of IUGR. Which of the above most accurately describes the situation, remains to be elucidated.

We had also previously observed (in IUGR placentas) an up-regulation of TR proteins, compared to normal gestational age matched controls; which could reflect potential compensation for

the low fetal circulating levels of TH, or be a contributing factor to the pathophysiology (Kilby *et al* 1998).

Therefore, further investigation is required in order to understand the roles of these proteins, particularly MCT8 and MCT10, in placenta, as well as the link between normal expression/function and malplacentation syndromes such as IUGR.

Models for Trophoblast Investigation

The study of human trophoblast function and the physiological processes associated with human implantation thus far has been *in vitro,* using primary cultures or human trophoblast derived cell lines (Kilburn *et al* 2000). From these studies we have learned a great deal about the factors which potentially contribute to the normal development and function of the human placenta. *In vivo* studies thus far using animal models such as rat and mouse, have been very important, but have highlighted significant interspecies variations in trophoblast specialization and utero-placental morphogenesis (Wooding *et al* 1994). However, specific classes of mutant phenotypes have been observed in the placenta of knock out mice, which resemble phenotypes associated with IUGR that are observed in humans. Thus, the potential for mutant mouse strains to be used to model the different clinical conditions of human pregnancy is encouraging (Cross 2000).

Due to limitations in availability of primary EVTs from first trimester placenta, and difficulty with performing long-term culture of homogeneous human trophoblast cell populations, the use of transformed primary cell lines forms a very necessary approach, particularly as *in vivo* work in humans is not possible (Liu *et al.* 2007).

HTR-8/SVneo is a first trimester human EVT like cell line; cultured from chorionic extra villous explants transfected and immortalised with the simian virus 40 large T antigen. The resulting cell line, HTR-8/SVneo, was found to exhibit morphological similarity and to share phenotypic features with the primary EVT parental cell cultures, including expression of cytokeratin (to confirm trophoblast identity) and secretion of 72-kDa type IV collagenase.

Similarity in the regulatory trends in response to treatment with signalling molecules such as transforming growth factor-β (TGF-β) were observed with respect to the invasion, migration and proliferation of both these cells and their progenitors *in vitro* (Graham *et al* 1993). Apart from the ability to sustain prolonged growth in culture, transfected HTR-8/SVneo cells were demonstrated to be phenotypically similar, and therefore important tools for the study of EVT function. HTR-8/SVneo cells have since been used as such in a number of studies (Graham *et al* 1993; Liu *et al* 2007; Jovanović *et al* 2010).

HTR-8/SVneo was selected as the trophoblast model for this study on the basis of the spontaneously invasive phenotype, and low expression of endogenous *MCT10* relative to other cell lines (average Ct value 34, compared to 27, 30 and 32 in JEG3, BeWo and SGHPL-4 respectively). Upon previous investigations of the phenotype of these cells, our group were also able to observe increased invasion in response to T3 (unpublished data).

Our work with this cell line however, as with the other trophoblast cell lines such as JEG3 and SGHPL-4, has highlighted the issue of variation in cell responses. Such differences in cell lines had been previously investigated by Lash *et al* (1999; 2007). Initial characterisation of these cell lines (immunohistochemistry) revealed that they did not express all of the markers that EVTs normally express (i.e. integrins α1, α6 and β3, cytokeratin-7 and HLA-G). The cellular responses of these lines also varied greatly, depending on the duration and conditions of culture. The application of cell lines for the study of trophoblast function is therefore, an important tool, although it is not a straightforward case of extrapolating back from experimental data on *in vitro* experiments, to EVT behaviour *in vivo*. Perhaps with the use of animal models and primary cell culture, a greater insight into EVT function can be anticipated in later studies.

Hypothesis and Aims

From the currently available data we can surmise that inadequate invasion and remodelling of spiral arteries occur in association with

IUGR in the vast majority of cases. IUGR remains a condition of heterogeneous aetiologies, in which the exact mechanisms responsible for its pathophysiology remain unclear, although these mechanisms are likely to be different for various primary pathologies. It *is* apparent however, that factors regulating the balance between trophoblast proliferation and death may play a significant role in all cases (Whitley *et al* 2007). T3 has been postulated to regulate the function of trophoblasts, while MCT8 and MCT10 are believed to be effective transporters of TH and important facilitators of the biological activity of TH. In light of where the research trail has arrived, we proposed the following hypotheses:

i) Both transporters play a major role in the delivery of TH into trophoblasts, which in turn impacts upon trophoblast activity and therefore on placental development.
ii) Altered placental expressions of MCT8 and MCT10 may contribute to the development of malplacentation syndromes resulting in poor fetal growth as in IUGR.

Therefore, the following aims were considered for this study.

Using HTR8/SVneo as a model of first trimester EVTs;

i) To determine the effect of T3 on the proliferation of EVTs,
ii) To determine the effect of T3 on the expression of; TH transporters MCT8 and MCT10, Deiodinases 2 and 3 (D2 and

iii) To determine the effect of altering MCT8 and MCT10 expressions on trophoblast function and whether these changes are T3 dependent.

2. Materials & Methods

Plasmids

The expression vector for the human MCT10 cDNA (pcDNA3.1+ hMCT10) and human MCT8 cDNA were kindly provided by Prof. T. J. Visser (Friesema *et al* 2008). The expression vector for the human MCT8 cDNA was haemagglutinin (HA) tagged at the C-terminus previously by our laboratory (pcDNA3.1+ hMCT8-HA) (Friesema *et al* 2006; James *et al* 2009).

Cell lines

HTR-8/SVneo cells were cultured in RPMI 1640 media containing 1 % benzyl-penicillin, 1% L-Glutamine and 10 % Fetal Bovine Serum (FBS). All reagents are from Sigma-Aldrich, unless otherwise stated. For T3 sensitive assays, 10% charcoal stripped FBS (steroid hormone/TH depleted) serum was used instead of 10% FBS and will be referred to as stripped media below.

T3 Response: Cell Proliferation

HTR-8/SVneo cells were plated (2.5 x 10^3 cells) in 96 well cell culture plates in charcoal stripped (steroid hormone and TH deplete) media consisting of RPMI, 1 % benzyl-penicillin, 1% L-Glutamine, and 10 % charcoal stripped FBS. Cells were cultured overnight and treated with fresh media containing 0, 0.1, 1, 5, 10 or 100nM T3. T3 was prepared at the relevant concentrations from a stock solution of

30µM T3 dissolved in DMEM (Invitrogen), supplemented with NaOH. Cells were cultured for up to 96 hours at 37° C, and 5% CO_2.

The Methyltetrazoleum (MTT) quantitative colorimetric assay was used to determine cell proliferation at times 24, 48, 72 and 96 hours of culture post treatment with T3. At each time point, cells were incubated with MTT reagent for 3h at 37° C, and lysed subsequently, by the addition of 100µl Dimethylsulf-oxide (DMSO) to each well as previously described by Barber *et al* (2005). Cell numbers were determined spectrophotometrically using the multi-well scanning spectrophotometer at optical density (OD) 545nm. Normal cell growth was established in each population of non treated cells (in 0nm T3, in stripped media), and compared with treated cells. Each treatment condition was performed in five replicates within an experiment, and each experiment was performed four times.

T3 Response: Messenger RNA (mRNA) Expression of Genes

HTR-8/SVneo cells were seeded in six well culture dishes in stripped media, and allowed to adhere to the dish overnight. The media was replaced with fresh media supplemented with or without T3 [0 or 10nM]. Total RNA from cells was extracted using TriReagent® (500µl) at times 0, 2, 6, 12, 24, and 48 h after T3 treatment, and stored at - 80° C. Protein was also harvested using 250µl of 2% SDS and also stored at - 80° C until analysis (n=5 experiments).

RNA Extraction and RT-PCR

RNA extraction and reverse transcription (RT) were performed as per manufacturer's guidelines and as previously described (Barber *et al* 2005). Chloroform (100µl) was added to cell lysates in TriReagent®, before centrifugation at 1200g at 4°C. 250µl of Isopropanol was added to the aqueous layer. Samples were centrifuged and supernatants subsequently removed. The pellets were washed with 500µl of 75% ethanol, vortexed and centrifuged at 7500g at 4°C for 10 minutes. Ethanol was aspirated carefully and pellets left to dry at room temperature before resuspension in 32µl of nuclease free water. RNA concentration and purity were determined using the NanoDrop® (Thermo Fisher Scientific) spectrophotometer, by measuring the absorbance ratio of RNA samples at 260 and 280 nm.

Total RNA was reverse transcribed with Avian Myeloblastosis Virus (AMV) Reverse Transcriptase using the Reverse Transcription system kit (Promega, Maddison WI, USA). The total RNA (0.5 µg) was made up to a total volume of 9.75 µl using nuclease free water and incubated at 70°C for 10 minutes. It was then mixed with $MgCl_2$ (5 mM), 1 x reverse transcription buffer (10 mM Tris-HCl [pH 9.0 at 25°C]; 50 nM KCl; 0.1% Triton® X-100), 0.5 µg of random hexamer primers, 4 mM of deoxynucleotide triphosphate (dNTP) mix, 20 units of ribonuclease inhibitor (RNasin) and 15 units of AMV reverse transcriptase in a total reaction volume of 20 µl. The reaction was incubated at room temperature for 10 minutes, and then at 42°C for

60 minutes. Samples were finally heated at 95°C for 5 minutes and at 4°C for 5 minutes.

Semi-quantitative TaqMan® PCR

TaqMan® Polymerase Chain reaction (PCR) was carried out in duplicate in a reaction volume of 25 µl in 96 well plates; the reaction buffer contained 1x TaqMan® Universal PCR Master Mix (Applied BioSystems), 150 nM TaqMan® probe (5' FAM / 3' TAMRA labelled; Eurogentec, UK) and 900 nM primers (Alta Bioscience, UK). Each probe and primer set was designed with Primer Express v2.0 (Applied BioSystems; **Table 1**) All the Primers and probes had previously been validated alongside control reverse transcription reactions without AMV (Chan *et al* 2002; Chan *et al* 2003 placenta; Chan *et al* 2003 NT2 cells; Loubiere *et al* 2010). All samples were also analysed for 18S rRNA (housekeeping gene) with a VIC-labelled-probe and primers (Applied BioSystems) as an internal control to correct for differences in RT efficiency.

Ct value and relative quantification of each gene was determined using the delta-Ct method as we have previously described (Chan *et al* 2006; Loubiere *et al* 2010). For each sample, fold changes in gene expressions relative to a calibrator were calculated with the equation $2^{-\Delta Ct}$ (ΔCt = sample Ct minus calibrator Ct). The calibrator was determined for each gene as the sample with the highest expression. For each sample the gene expression was then normalised to 18S expression.

Basal Expression (mRNA) of TH Transporters

The basal expression of endogenous MCT8 and MCT10 as well as other TH transporters was investigated in HTR-8/SVneo cells, to compare TH transporter expression with other cell lines (mRNA expression (ΔCt values) had been determined by our group previously in other cell lines). The other transporters investigated were OATPE, LAT1, LAT2, and its obligate hetromer 4F2Hc. HTR-8/SVneo cells were cultured in RPMI media for 24h, before RNA was harvested (in the same way as described previously using TriReagent® (500µl)). Total RNA was reverse transcribed and the Q-PCR method was used to determine ΔCt values for the expression of each transporter as previously described.

Altering MCT8/MCT10 Expression

For over-expression studies, HTR-8/SVneo cells were transfected with either WT human MCT10 or human MCT8. For suppression of MCT8 expression, cells were transfected with small interfering RNA (siRNA) (Silencer® pre designed siRNA from Ambion) targeted at MCT8. The MCT8 siRNA targeted a region in the third exon of the MCT8 gene. Non-targeting or scramble siRNA (Silencer Negative control; Ambion) was used as a negative control (**Table 2**), both using the Nucleotransfection® technology from Amaxa which transiently induces destabilisation of cell membranes and allows cellular entry of nucleotides for effective transfection into cells.

Gene	Forward primer	Reverse primer	Probe
MCT8 (NM_006517)	5' -CAA CGC ACT TAC CGC ATC TG- 3'	5'-GTA GCC CCA ATA CAC ACC AAG AG-3'	5'-TCC ACA TAC TTC ATC AGG TGT ACA TAG GGA ACA AA-3'
MCT10 (NM_018593)	5'-GAT TCA TGT CTA TAC CCA TGA CTG TTG-3'	5'-CAC ATC ATA GGA GCC CAG TTT GT-3'	5'-CCC ACC CAT TGC AGG GTT ACT TCG T-3'
D2	5'-CTG CGC TGC GTC TGG AA-3'	5'-GAG ACA TGC ACC ACA CTG GAA T-3'	5'-CCC AAT TTC ACC TG TTT GTA GGC ATC GAG-3'
D3	5'-GCT TCC AGA GCC AGC ACA TC-3'	5'-GCT GCC GAA ATT GAG AAC CA-3'	5'-TCG ACT ACG CGC AAG GGA ACC G-3'
TRb1	5'-GTG TCT CAA GTG CCC AGA CCT T-3'	5'-CAC AGA GCT CGT CCT TGT CTA AGT AA-3'	5'-TGG GGA TGT ACC CTT TAC ATT TCT TCT CC TCC-3'
Cx43 (NM_000165)	5'-TTC ATT TTA CTT CAT CCT CCA AGG A-3'	5'-CGC TCC AGT CAC CCA TGT T-3'	5'-CCT GGG CAC CAC TCT TTT GCT TAA AAG TAGTG-3'

Table 1. The quantitative primer and probe sequences. Each pair of primers was designed to span exon–intron boundaries to prevent genomic amplification, except for deiodinase type 3, which is a single exon gene.

Sequence	Scramble siRNA:	MCT8 siRNA:
Sense	5'-GGGCCACAGUUUCAGCUUCtt	5'-GCCUGCGCUACUUCACCUAtt
Antisense	5'-GAAGCUGAAACUGUGGCCCtt	5'-UAGGUGAAGUAGCGCAGGCtt

Table 2. Silencing RNA sequences directed at MCT8.

Cells were harvested and counted. One million cells were used per reaction. Cells were centrifuged at 1250 rpm (in a swing-out rotor, bench-top centrifuge, Hettich; at approximately 245g) for 10 minutes, and then re-suspended in Nucleofection® solution V (100µl per reaction), for transfection with hMCT10, hMCT8, pcDNA3.1+ vector only or siRNA targeted at MCT8. 100µl of cell suspension was mixed with the relevant concentration of either vector cDNA (2µg)

or siRNA (30nM), for electroporation using the Amaxa Nucleofector® L109 programme (as optimised by our group for good transfection efficiency and cell viability, unpublished data).

Electroporated cells were resuspended in RPMI media and seeded in either 6 well plates, clear or white walled 96 well plates for RNA/protein extraction or MTT/Caspase assays respectively. Cells were also plated in 6 well plates after electroporation, to fix for fluorescence activated cell sorting (FACS). Cells were cultured overnight at 37° C, and 5% CO_2. Stripped media was used to replace media at 24h of culture. Cells were washed again with PBS after 4 hours, and cultured in media with or without 10nM T3. All subsequent experiments were performed at 48 hours post-T3 treatment (but 72 hours after culture) to allow time for complete down-regulation of protein, and protein stabilisation (Barber *et al* 2005; James *et al* 2008).

Western Immunoblotting

Western blots were used to validate over expression of MCT8 and MCT10 and the silencing of MCT8 at the protein level. Proteins were harvested using 250µl 2% SDS from HTR-8/SVneo cells seeded in six well plates. Protein concentration was determined via the Bradford assay, with BSA as a standard, after which samples were subsequently denatured in loading buffer with DTT (54mg/ml) at room temperature for an hour. Protein was loaded at 30µg or 70µg into 8% SDS/polyacrylamide gels in an electrophoresis tank and

electrophoresed for 2h in running buffer (first at 70V, then at 150V) as previously described (Loubiere *et al* 2010).

Proteins were then transferred onto poly-vinylidene fluoride (PVDF) membrane for 1.5 hours at 360 mV. Non-specific protein binding on the membranes was blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST), at room temperature for an hour. Membranes were washed with TBST and then incubated with primary anti-rabbit polyclonal MCT8 (Sigma, Genosys) or MCT10 antibody (Charles Rivers, Edinburgh), at 3 x 10^{-3} mg/ml, and 1.294 x 10^{-3} respectively, in 5% milk in TBST overnight at 4 °C. Antibody specificity was validated by our group as previously described for MCT8 (Loubiere *et al* 2010), targeted to the intracellular region in PEST region, towards the N-terminus; to the sequence SQASEEAKGPWQEA[C]-KLH. Specificity for MCT10 was also validated by our group to be targeted to the following sequence at the C-terminus; SSGMFKKESDSII, see **Figure 2.1**.

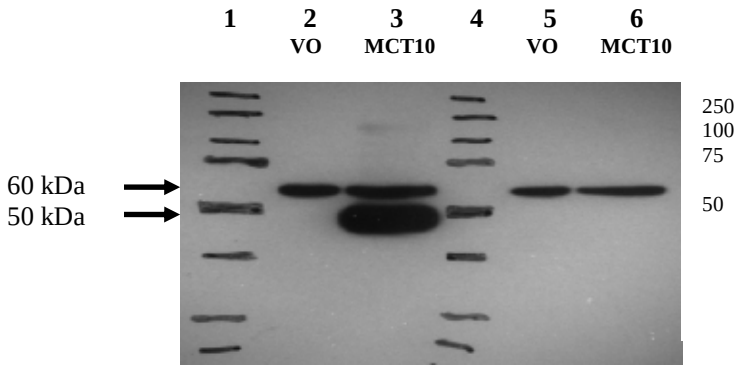


Figure 2.1. Western blot of protein from HTR-8/SVneo transfected with VO and wtMCT10 (left to right). Protein was incubated with MCT10 Ab (lanes 2 and 3) at 1:3000, and MCT10 Ab with blocking peptide, (lanes 5 and 6) at 15X more than Ab. Protein bands can be seen at 50 and 60 kDa, but specificity appears to be at 50 kDa.

The membranes were then washed and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase in 5% milk in TBST, at room temperature for an hour. Membranes were washed and bands visualised using the ECL Chemi-luminescence detection system (GE Healthcare Life Sciences, Buckinghamshire). Protein migration and subsequent size was compared against the Hyper-ladder precision plus standards (BioRad). Monoclonal primary anti-mouse β-actin antibody (Sigma A5441) at a 1:2000 dilution was used on the same membrane to evaluate protein loading.

Apoptosis; Caspase-Glo® 3/7.

Caspase-Glo® (Promega) is a luminescence assay that determines the levels of Caspase 3/7 activity. It was used as described in the manufacturer's manual (Promega Technical Bulletin Caspase-Glo® 3/7 Assay: Instructions for use of products). Cells were cultured in white walled 96 well plates and the media replaced with stripped RPMI media, 24 hours after transfection. Media was replaced again with stripped media 4 hours later. For the reaction, Caspase-Glo® 3/7 Substrate was reconstituted in Caspase-Glo® 3/7 Buffer and 100µl added to each well in the dark (1:1 ratio of media to reagent) at 48h (post T3 treatment) of culture. The plate was shaken and incubated at room temperature for an hour in the dark. The plate was then read on a multi-well scanning spectrophotometer for luminescence at 590nm. The level of luminescence observed corresponds to the level of Caspase 3/7 activity.

Statistical Analysis

Aanalysis was performed using SigmaStat® 3.1. software, using the Two Way ANOVA, with Holm-sidak post hoc tests. Significance was taken as $p<0.05$.

3. Results

Effect of T3 on Cell proliferation

The effect of T3 on the proliferation of HTR-8/SVneo cells was assessed over 96h via the MTT assay. As shown in **Figure 3**, proliferation of HTR-8/SVneo cells occurred both in the absence and presence of T3. We were able to show that T3 treatment had no effect on the proliferation of HTR-8/SVneo cells during this time.

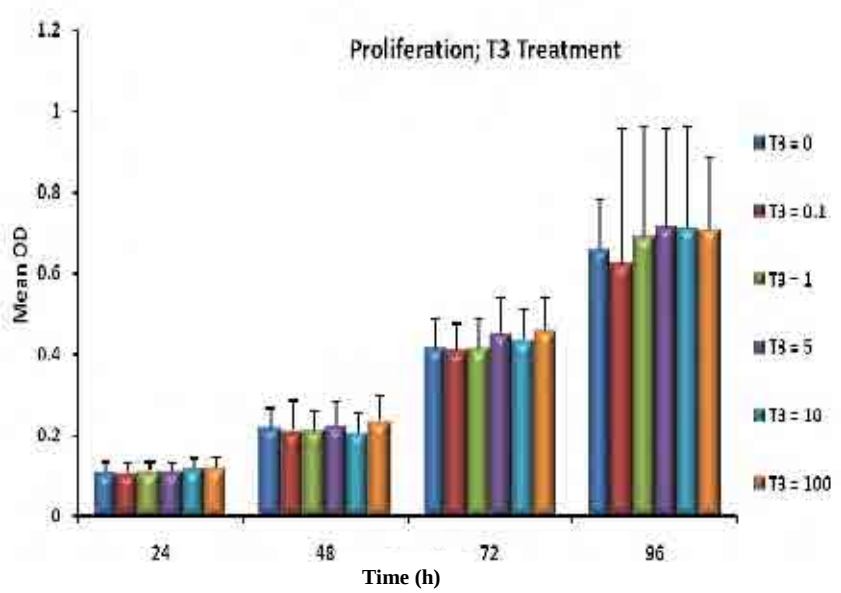


Figure 3. Proliferation of HTR-8/SVneo cells over 96h, in absence and presence of 0 – 100nM T3. Cell proliferation was measured spectrophotometrically by the MTT assay (n=4 experiments).

Basal mRNA Expression of TH Transporters

The basal expression of each of TH transporters (MCT8, MCT10, LAT1, LAT2, 4F2hC and OATPE) was determined in HTR-8/SVneo using Q-PCR, and compared to previously determined values (Vasilopoulou, unpublished data) in other cell lines, and whole placenta, **Table 3.** HTR-8/SVneo cells have a lower mRNA

expression of MCT8 (30 cycles) relative to SGHPL-4 (26 cycles) and term placenta (29 cycles), and a higher expression compared to JEG-3 (36 cycles), BeWo (35 cycles) and JAR cells (36 cycles). MCT10 has a higher mRNA expression (31 cycles) relative to SGHPL-4 (32 cycles) but a lower expression relative to term placenta (28 cycles), JEG-3 (27 cycles), BeWo (30 cycles) and JAR cells (26 cycles).

	Average ΔCt					
Transporter	Term Placenta	HTR-8/Svneo	JEG-3	BeWo	SGHPL-4	JAR
LAT1	27	26	19	20	23	20
LAT2	27	34	20	21	31	27
4F2Hc	26	28	21	21	24	21
OATPE	26	29	23	23	29	22
MCT8	29	30	36	35	26	36
MCT10	28	31	27	30	32	26

Table 3. The comparative expressions of mRNA encoding various TH transporters in term placenta, JEG-3, BeWo, SGHPL-4, JAR cells (Vasilopoulou unpublished data) and in HTR-8/SVneo (present study) via RT (of total RNA) and Q-PCR. Cells were not treated with T3, and expression is expressed as average ΔCt values.

Effect of T3 on Gene Expression.

The effect of T3 on gene expression was measured by real time Q-PCR, **Figure 3.1 and 3.2**. There was a significant decrease observed in the expression of *TRβ1* with T3 treatment (Two way ANOVA effect of T3 p =0.036), but no change in that of *Connexin43*, D2, D3 or *MCT10* and *MCT8* expression with T3.

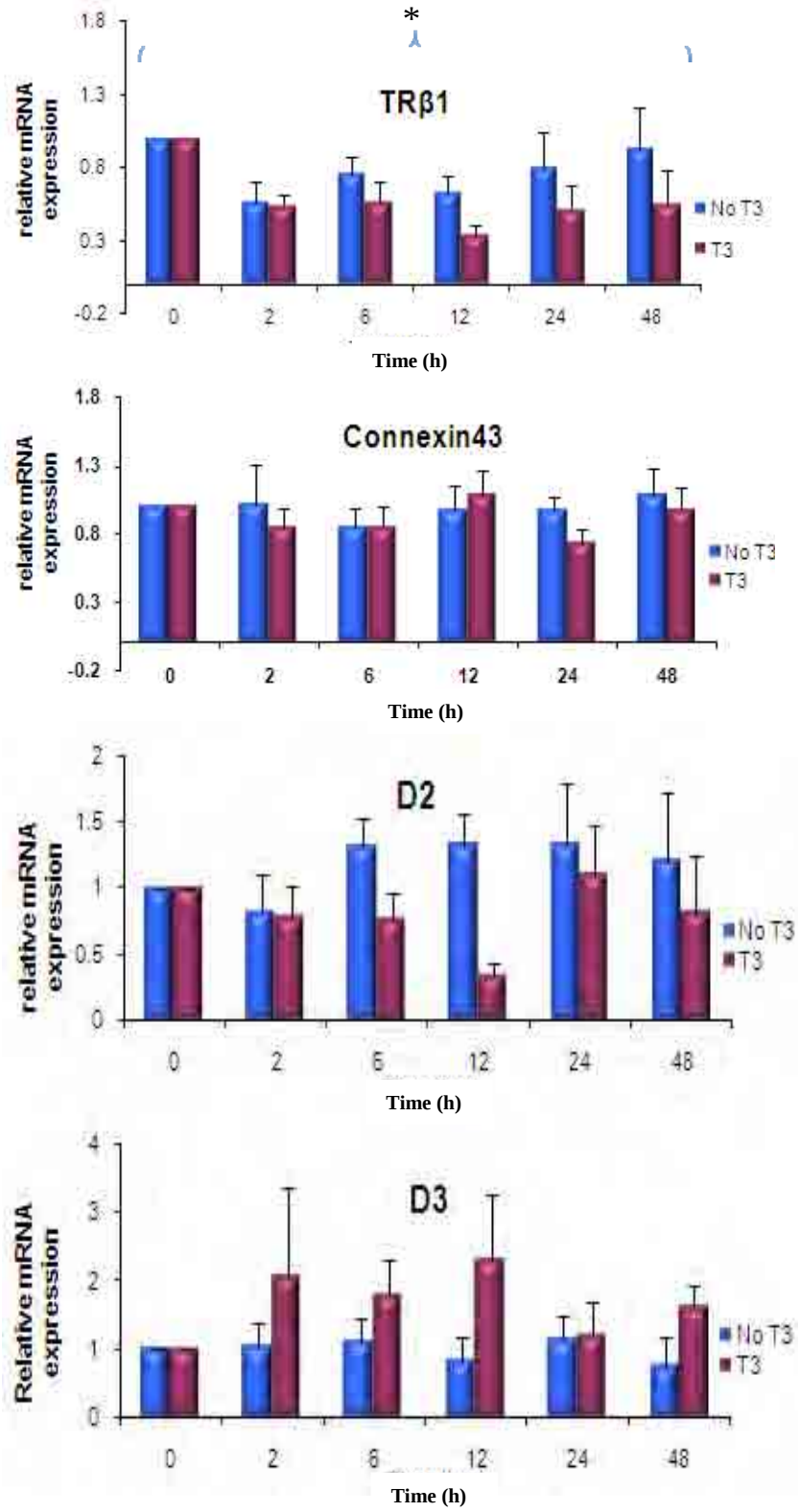


Figure 3. 1. The relative expression of mRNA encoding *TR-β1*, *Connexin43, D2* and *D3* in HTR8-SVneo cells treated with (+/-) 10nM T3; determined at 0, 2, 6, 12, 24 and 48h of T3 treatment (compared to cells treated with 0 T3, at time 0 of T3 treatment) by RT-PCR. mRNA expression at 0h was given an arbitrary value of 1 (n=5). *** p=<0.05**

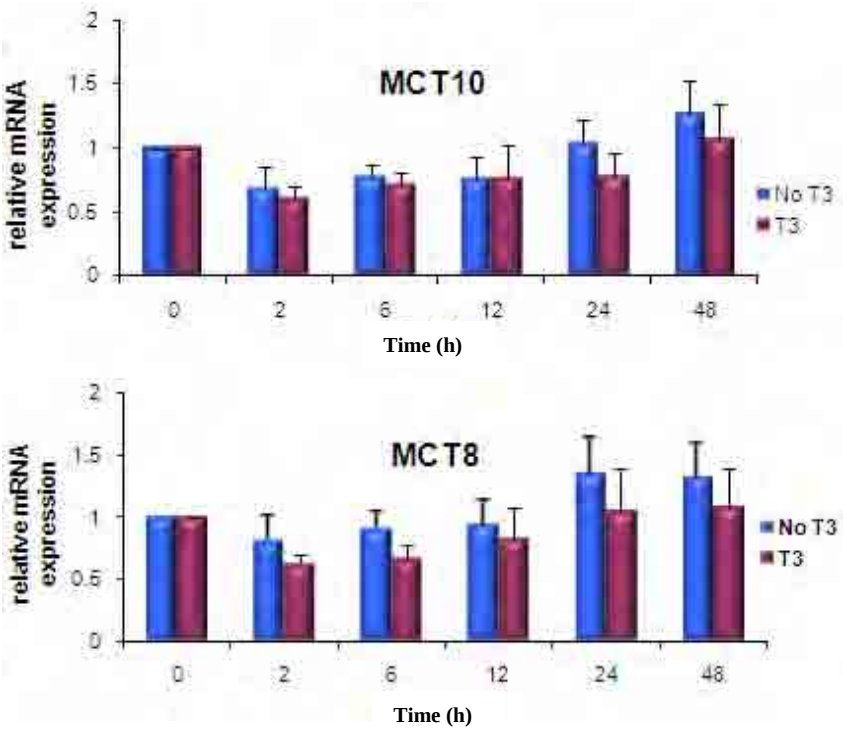


Figure 3. 2. The relative expression of mRNA encoding *MCT10* and *MCT8*, in HTR8-SVneo cells treated with (+/-) 10nM T3, determined at 0, 2, 6, 12, 24 and 48h of treatment (compared to cells treated with 0 T3, at time 0) by RT-PCR. mRNA expression in cells at time 0 was given an arbitrary value of 1 (n=5).

Confirming Alterations in the Expression of MCT8 and MCT10 in HTR-8/SVneo

Over-expression of MCT8 was confirmed at the protein level (n=4) via fluorescence activated cell sorting (FACS), where cells transfected with MCT8 were compared to control cells transfected with vector only (VO) for the expression of MCT8. The FACs data from a representative experiment is shown in Figure 3.3, where 71% and 76% of cells (treated with 0 and 10nM T3 respectively) were shown to express exogenous MCT8 (as determined by FACs for HA), thereby revealing a good transfection efficiency and successful over-expression of MCT8. Over-expression was also confirmed via western blotting **Figure 3.3** where the MCT8 protein bands were

observed at approximately 60KDa (compared to VO controls) as previously demonstrated for MCT8 (Loubiere *et al* 2010). MCT10 over-expression was confirmed via western blot (**Figure 3.4)**, where MCT10 protein bands were observed at approximately 50KDa. The specificity of the MCT10 antibody and the subsequent band observed on a western blot was validated by our group previously as outlined in the methods.

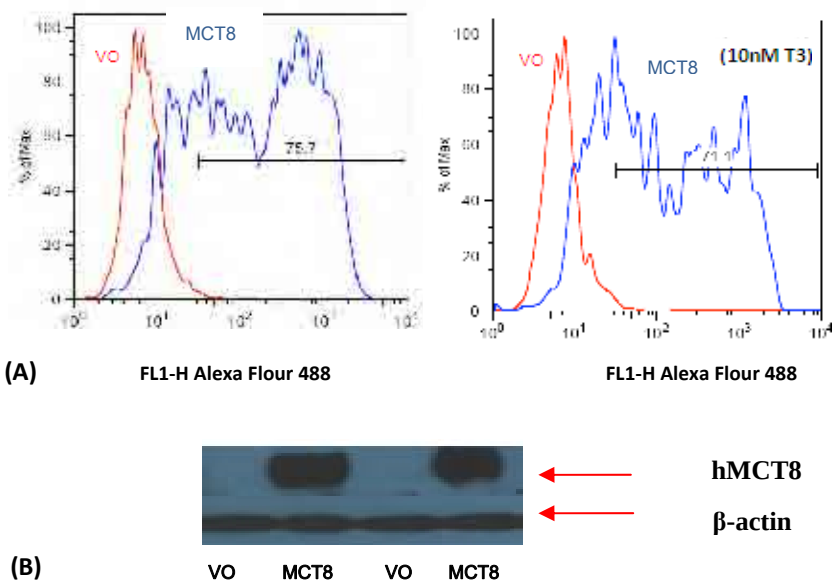


Figure 3. 3. Validation of over-expression of MCT8 at 72h post transfection. **(A)** Fluorescence activated cell sorting (FACS) for MCT8 transfected cells compared to vector only (VO) cells (± T3). One representative experiment is shown **(B)** Western Blot of MCT8 (60KDa) protein (30µg protein loaded) expression in cells transfected with MCT8 compared to VO (as shown for one representative experiment).

Figure 3. 4. Validation of MCT10 overexpression via Western Blot at 72h post transfection. MCT10 overexpression is observed; as determined in total protein from cells transfected with MCT10 (approximately 50KDa protein), compared to VO controls after loading 30µg of protein into each lane.

Silencing of MCT8 (i.e cells transfected with siRNA vs scRNA control) was confirmed at the mRNA level by RT-PCR. An average of 67% silencing was achieved overall **Figure 3.5**. However, silencing could not be demonstrated at the protein level (via western blotting). Detection of endogenously expressed MCT8 via western blotting, was only possible with greater concentrations of protein loaded (70µg rather than 30µg), as we observed no bands at the usual concentration of 30µg (VO lane of western blot **Figure 3.3.B**). Therefore, for comparison of MCT8 expression in cells silenced for MCT8, a greater concentration of protein was loaded (70µg), but there was no observable reduction in the expression of MCT8, even following overnight exposure of the film (**Figure 3.5)**.

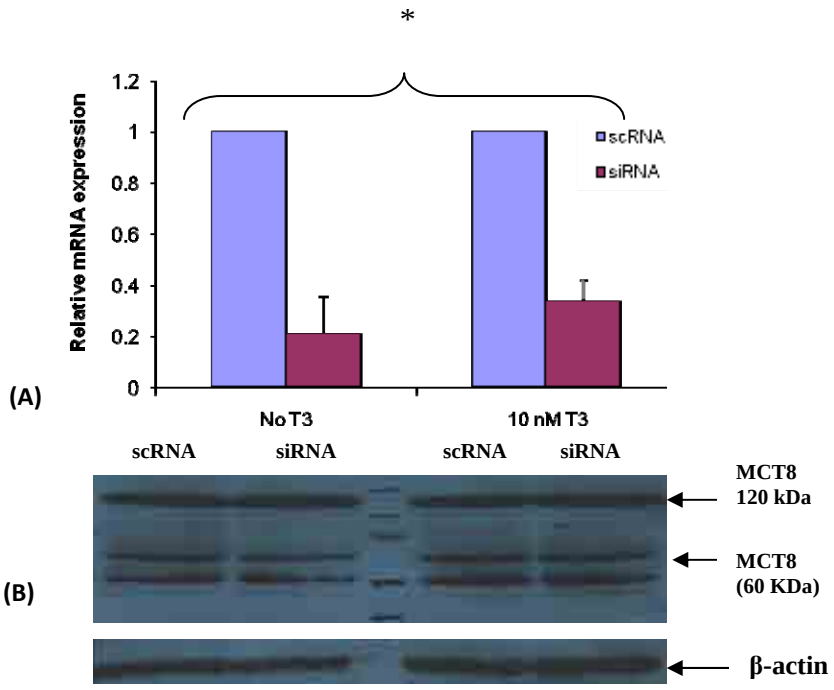


Figure 3. 5. Validation of silencing of MCT8 at the mRNA and at the protein level at 72h post transfection. **(A)** Silencing of MCT8 determined via RT-QPCR, an average of 73% silencing was observed for cells transfected with silencing RNA (scRNA), in cells treated +/-T3. **** p = 0.05.*** **(B)** Expression of endogenous MCT8 protein (60KDa, 120kD) after silencing MCT8 compared to controls (siRNA vs scRNA). Bands were visible after loading 70µg protein (rather than 30µg), and exposing the film overnight.

Both the over-expression and silencing were assessed at 72h post transfection, but 48h post T3 treatment (± 10nM T3).

Effect of Altered MCT8/MCT10 Expression on Cell Proliferation

The effect of over-expressing MCT8 and MCT10 (compared to control cells transfected with VO) on cell proliferation was investigated (**Figure 3.6**) at 48h post T3 treatment (± 10nM T3). MCT8 transfection resulted in a significant reduction in cell proliferation compared to vector only (ANOVA effect of MCT8 *p = 0.005*); post hoc tests revealed this reduction of proliferation to be significant only in the presence of T3 ($p < 0.01$). There was a 36 % reduction in proliferation in MCT8 transfected cells compared with cells transfected with vector only in the presence of T3. Similarly, MCT10 transfection led to significant reduction in cell proliferation compared to cells transfected with vector only (ANOVA *effect of MCT10 $p < 0.05$);* although post hoc analysis revealed this effect to be significant only in the presence of T3, where a 29% reduction in cell proliferation was observed ($p < 0.05$). Conversely, a trend of increased cell proliferation was observed after silencing MCT8 (at 48h of culture post T3 treatment), although this trend was not statistically significant (ANOVA $p = 0.166$) (**Figure 3.7)**.

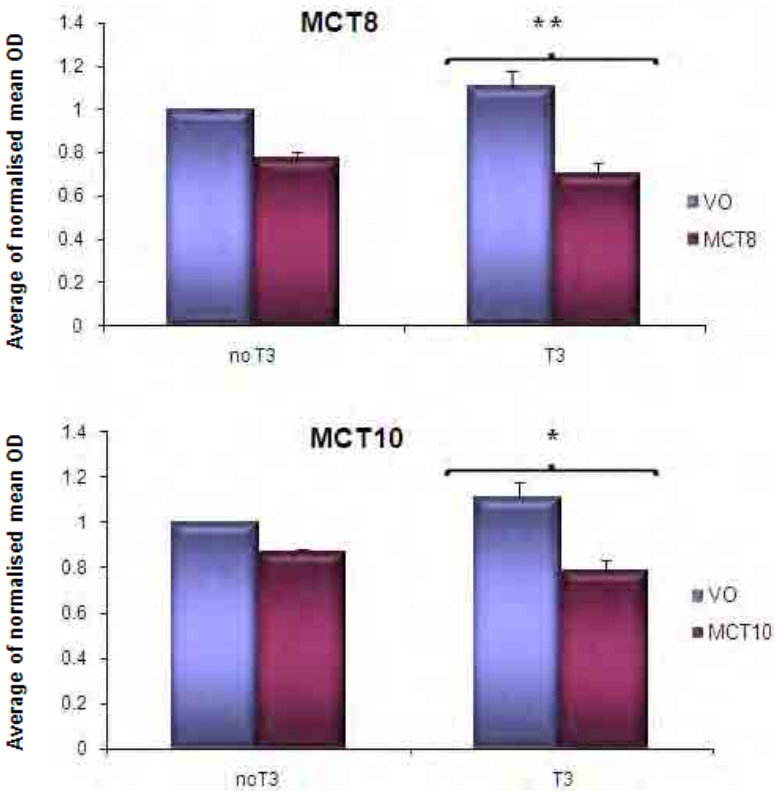


Figure 3.6. Cell proliferation (assessed by MTT measured 48h after treatment with T3) was significantly reduced in response to overexpression of MCT8, and MCT10 in HTR-8/SVneo cells only in the presence of T3 (10nM). Pooled data from n = 4. Within each experiment, the mean OD at 545nm for each group was normalized to that of VO transfected cells cultured in 0 T3nM, (which was given an arbitrary value of 1), before pooling these values, from n=4. (***p<0.05, ** p<0.01)**

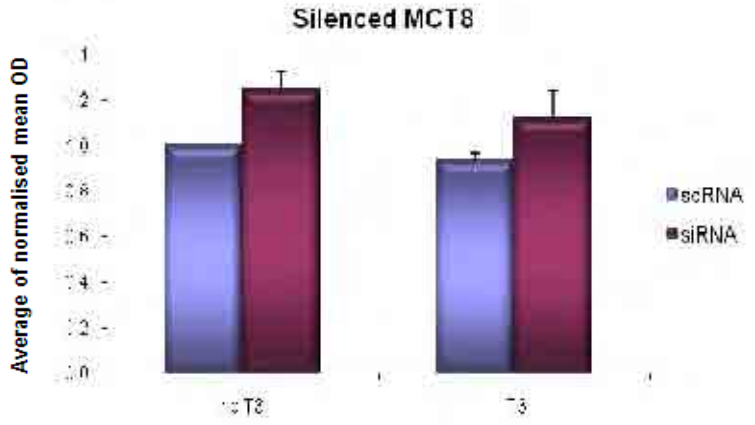


Figure 3. 7. Cell proliferation as assessed by MTT at 48 h post T3 treatment of HTR-8/SVneo cells transfected with small interfering RNA (siRNA) targeted at MCT8 compared to cells transfected with control scramble RNA (scRNA). Mean OD for each group was normalized to that of scRNA transfected cells cultured in 0 T3nM, which was given an arbitrary value of 1. (n = 4).

Effect of Altered MCT8/MCT10 Expression on Apoptosis

The Caspase 3/7 activity assay was used to determine the level of the pro-apoptotic markers, Caspase 3 and 7, in response to the over-expression of MCT8 and MCT10, and the silencing of MCT8, in the presence and absence of T3.

MCT8 over-expression was observed to produce a significant reduction (ANOVA: effect of MCT8 $p < 0.01$) by 24% and 31% in apoptotic marker detection in the absence and presence of 10nM T3, respectively. This effect was found to be independent of T3 in post hoc tests (which revealed significance both with ($p < 0.01$) and without ($p<0.03$) T3). Similarly, MCT10 over-expression significantly reduced apoptosis (ANOVA: effect of MCT10 $p < 0.05$) by 12% and 19% in the absence and presence of 10nM T3 respectively, again an effect that was significant independently of T3 (**Figure 3.8**).

Conversely, following MCT8 silencing, Caspase 3/7 activity was significantly increased (ANOVA: effect of MCT8 $p<0.01$); by 89% and 34.5% in the absence and presence of 10nM T3 respectively, compared to the control (scRNA transfected) cells (**Figure 3.8**). Post hoc tests revealed the increase in apoptosis to be independent of T3, i.e. that this effect was significant both in the absence ($p=0.01$) and presence ($p=0.036$) of T3.

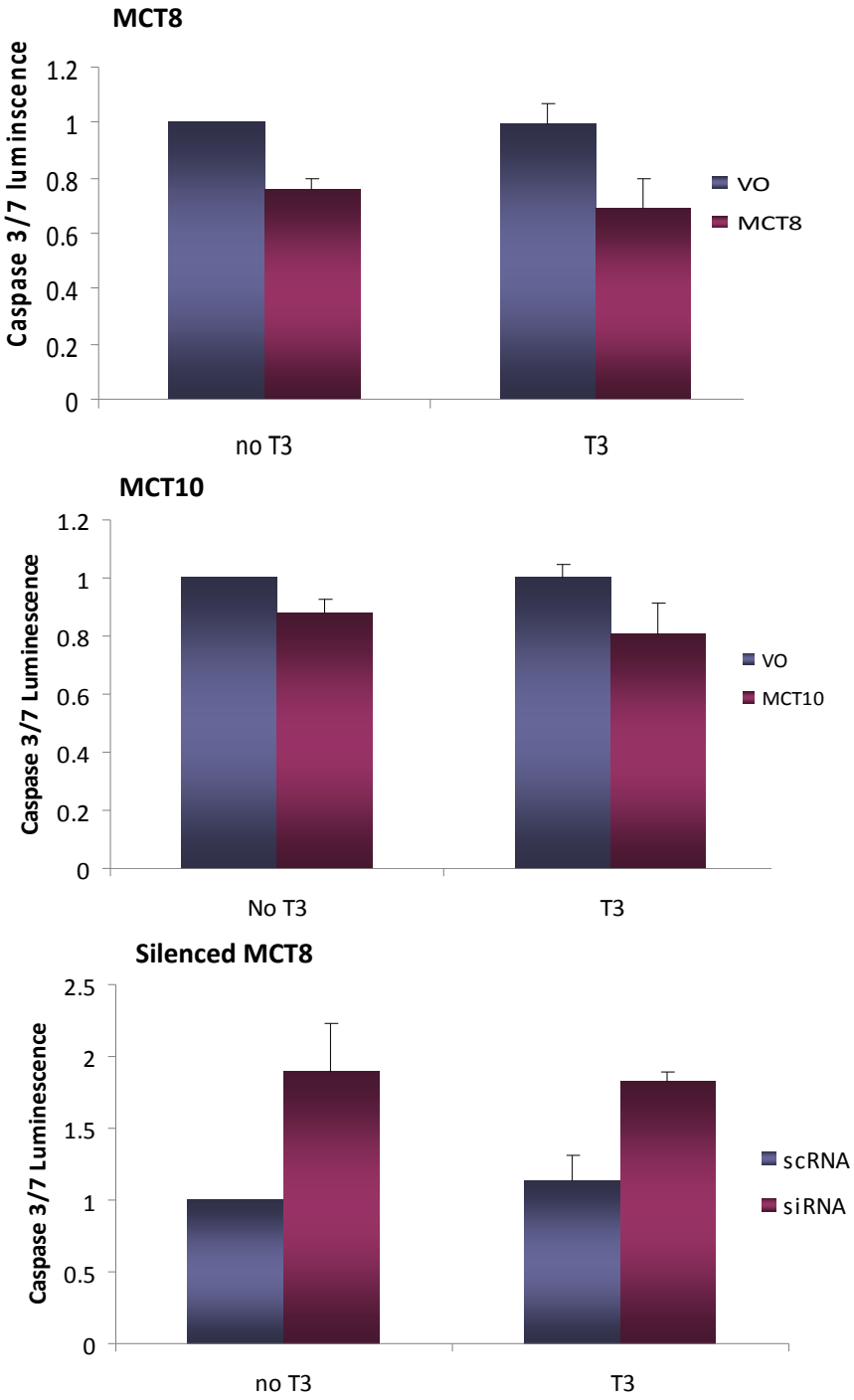


Figure 3.8. Caspase 3/7 activity in cells; over-expressing MCT8 (n=4) and MCT10 (n=3) compared to VO; MCT8 silencing compared to scRNA (n=3). Controls (VO or scRNA with no T3) were given an arbitrary value of 1.

Discussion

In this present study we have demonstrated for the first time that HTR-8/SVneo cells are T3 responsive and that the TH transporters Monocarboxylate transporter 8 (MCT8) and 10 (MCT10) are implicated in the modulation of proliferation and apoptosis, via T3 mediated and T3 independent pathways. These findings are consistent with our hypothesis that TH transporters play a critical role in the regulation of T3 mediated effects in EVTs. In addition, our findings demonstrate that MCT8 and MCT10 may also exert their effects independently of T3 transport.

T3 has previously been shown to regulate the proliferation, invasion, survival and differentiation of trophoblasts (Matsuo *et al* 1991; Kilby *et al* 1998; Lash *et al* 1999; Chan *et al* 2002; Barber *et al* 2005). The EVT is essential in the invasive component of placentation; its function believed to be regulated by many factors, including T3. Thus, the EVT has the cellular apparatus to be TH responsive (Matsuo *et al* 1991). In cases of pathology associated with abnormal TH levels, as in hypothyroid mothers, there are serious implications in placentation including considerably higher rates of placental complication (Davis *et al* 1988; LaFranchi *et al* 2005). Various studies have addressed the link between abrogated TH action and placental complication, in addition to the various factors influencing TH action, such as TH receptor isoforms (TRs) or Deiodinases. Our group and others have previously localised TRs and Deiodinases to the EVT in assessing the responsiveness of EVTs to T3 (Kilby *et al* 1998; Chan

et al 2003; Barber *et al* 2005). The exact pathways that control T3 responsiveness in the EVT have not yet been elucidated, although T3 could potentially be implicated directly or indirectly, in a number of pathways including cell signalling, mitochondrial gene transcription pathways, induction of [Ca^{2+}] or control of proliferation (Basset *et al* 2003).

We sought to investigate the potential role of T3 (by characterising EVT responsiveness to T3) and TH transporters MCT8 and MCT10, in an EVT-like cell line, HTR-8/SVneo. In the present study we found that cell proliferation was not affected by T3, which was a finding consistent with previous observations by our group in primary trophoblasts isolated from term placenta; where our group showed no change in viability in response to T3 (Barber *et al* 2005). We previously reported on an anti-proliferative effect of T3 in another EVT-like cell line, SGHPL-4, as well as a pro-proliferative effect of T3 in the choriocarcinoma line JEG-3; suggesting that EVT cell lines are differentially responsive to T3.

These data are consistent with wider observations reported in previous studies investigating T3 effect on proliferation, where both anti-proliferative and pro-proliferative effects of T3 have been reported in trophoblasts (Maruo *et al* 1991; Matsuo *et al* 1993; Barber *et al* 2005). The exact effect of T3 on proliferation depends on the cell or tissue type and developmental state. For instance, T3 has been reported to activate the proliferation of hepatocytes after

partial hepatectomy (Francavilla *et al* 1994) and induce the proliferation of cultured bovine thyroid cells (Di-Fulvio *et al* 2000), pancreatic acinar cells (Ohmura *et al* 1997; Ledda-Columbano *et al* 2005), bone marrow pro-B cells (Foster *et al* 1999), and of renal proximal tubular epithelial cells (Ohmura *et al* 1999). T3 also increases DNA synthesis in osteoblasts (Kassem *et al* 1993), and various other cell types. Conversely, T3 treatment may inhibit proliferation or cause anti-proliferative effects, as observed for oligodendrocyte progenitor cells or neuroblastoma N2a-β cells (Baas *et al* 1997; Garcia-Silva *et al* 2002). T3 may also prevent the cycling of mammary epithelial cells (Gonzales-Sancho *et al* 2002).

Much is yet to be understood about the exact regulatory actions of T3 in EVTs, although these investigations in other cell types offer insight into the potential ways in which T3 may exert its effect in the EVT. The lack of proliferative response in HTR-8/SVneo for instance, may possibly be an equal balance of both anti-proliferative and pro-proliferative effects, leading to no overall effect observed in cell number, unlike for the above cell types, where the positive or the negative effects of T3 pre-dominate.

On investigating the effect of over-expressing MCT8 and MCT10 on the proliferative response of HTR-8/svneo cells in the present study, we were able to demonstrate a significant reduction in proliferation, but only in the presence of T3. MCT8 silencing however, produced a

consistent trend (although not significant) of increased proliferation irrespective of T3 treatment.

Change in MCT8 and MCT10 Expression

The observations in the proliferation of cells over-expressing MCT8 and MCT10 are likely to be a consequence of raised T3 uptake and subsequent intracellular T3; as opposed to restoration of intracellular T3 to normal cellular levels when treating cells with T3 without over-expression of MCT8 or MCT10. In this scenario, T3 may regulate proliferation in a number of ways including through the control of the cell cycle (discussed later). However, as a similar trend of reduced proliferation (although not significant) was observed in the absence of T3, in cells over-expressing MCT8 and MCT10; these data suggest that effects may be independent of T3, pointing to other non-T3 dependent effects of MCT8 and MCT10. This is consistent with the findings of a previous study looking at the effect of MCT8 over-expression in NT2 and JEG-3 cells; where a T3 independent response of reduced proliferation was observed (James *et al* 2009).

The T3 independent effects observed in HTR-8/SVneo, and previously in JEG-3 and NT2 cells, point to alternate roles of MCT8 and MCT10 in cellular processes that affect proliferation. Little is known about which potential processes at present, but this is a potential future area of investigation, into the roles of MCT8 and MCT10 away from TH transport.

Although the trend of increased proliferation observed on silencing for MCT8 is not statistically significant, it reinforces the over-expression data. To either accept or reject this silencing trend as a true one, further N numbers should be anticipated. However, rather than experimental inconsistency, incomplete silencing of MCT8 may reflect why the trend observed is not significant. In this scenario compensation in TH transport by other transporters, may be responsible for limiting the effect of MCT8 silencing, perhaps by restoring cellular capacity to transport T3, and therefore restoring T3 regulation of proliferation. However, as the endogenous expression of MCT8 in HTR-8/SVneo is relatively low already, knockdown may not have a large effect in overall cell cycle control, and the effect is therefore not significant.

In order to validate this hypothesis, the expression (mRNA and protein) of cell cycle regulators, and MCT8 should be investigated after silencing for MCT8 as well as looking at the expressions of T3 responsive genes and the other transporters. Should the regulation of proliferation be via another pathway, the expressions of important cellular markers may be investigated, to try and pinpoint not only the magnitude of effect but also the mechanisms of possible T3/MCT8/MCT10 regulation of proliferation; not only after silencing for MCT8, but also after treating with T3 and/or over-expressing MCT8 and MCT10.

The mRNA expression of *MCT8* was determined after silencing in this study, to be 73% successful. This may account for the comparatively mild change in proliferation of HTR-8/SVneo cells, observed following MCT8 silencing (due to the persistence of MCT8 function). Silencing would need to be observed at the protein level, although this was not possible in the present study due to the already low levels of MCT8 expressed endogenously in this cell line.

Gene suppression following silencing is usually a transient phenomenon, as RNAi generally occurs within 24 h of transfection, and gene expression usually recovers 96 to 120 hours after transfection, although both onset and duration of RNAi depend on the turnover rate of the protein of interest, as well as serum dilution and longevity of the siRNAs (Mocellin *et al* 2004). Furthermore, the duration of gene silencing can be modified by factors such as the concentration of serum in the culture medium, which affects cell-cycle rate (Mocellin *et al* 2004). It is therefore necessary to determine the time course of any silencing observed under specific conditions and to ensure MCT8 silencing has been achieved at the time of proliferation or apoptosis assays, at both the mRNA and protein level. Therefore, assays should be repeated once silencing can be confirmed at the protein level.

It has been previously established in NT2s (James *et al* 2008) and in primary CTs (Vasilopoulou, unpublished data) that MCT8 knock down was achieved after 72 hours of culture. As MCT8 silencing in

HTR-8/SVneo had not been previously attempted, this time point was selected. Silencing was confirmed at this time point, to be at a satisfactory 73% average.

Regulation of Proliferation via the Cell Cycle

The major regulatory mechanism for cell proliferation is via the control of the cell cycle, as demonstrated in previous studies such as for GC cells (Barrera-Hernandez *et al* 1999). However, regulation may also occur via different cellular pathways. In a study using rat aorta smooth muscle (RASM) cells, proliferation was found to be regulated positively via the induction of NOX1 mRNA and protein, which is a mitogenic oxidase associated with increased proliferation (Wang *et al* 2010).

There are many genes and proteins that are central to cell cycle control; such as the retinoblastoma protein (Rb), the E2F family of transcription factors, cyclins, cyclin-dependent-kinases (cdks), cdk inhibitors and Mdm2. These regulators are all involved in the control of both cell proliferation and apoptosis (Barrera-Hernandez *et al* 1999; Puzianowska-Kuznicka *et al* 2006). Some of these factors have been found to be regulated by T3.

E2F transcription factors for instance are important in the regulation of G1 progression to S phase in the cell cycle. E2F1 in particular, is responsible for activating the transcription of a number of target genes that result in cell cycle progression. A negative TRE (as

observed in TSH promoters) was found in the E2F1 promoter indicating T3-TR regulation. Expression of E2F1 mRNA and protein was reported to be down-regulated by T3, leading to decreased proliferation due to cell arrest in G1 and S phases (Puzianowska-Kuznicka *et al* 2006). Cyclins have also been investigated for T3 regulation, leading to changes in cell cycle progression. Studies in Neuroblastoma cells (N2a-β) revealed indirect T3 regulation of the expression of cyclin D1 mRNA and protein via cyclic AMP response element (CRE) binding proteins (CREB) or activation transcription factor 2 (ATF-2) (Garcia-Silva *et al* 2004).

These genes and other markers for cell cycle control present as potential candidates for further investigation in HTR-8/SVneo cells, in order to ascertain the mechanism by which the potential T3 regulation of proliferation (reduction in proliferation in cells over-expressing MCT8 and MCT10 significant only in the presence of T3) may occur in cells over-expressing TH transporters. It could be that MCT8 and MCT10 may preferentially divert T3 effects down particular T3-response pathways within cells, to promote the anti-proliferative effect observed. The exact mechanisms of T3 regulation are unclear in the EVT, yet there are examples of both genomic and non-genomic regulation in other cell types (Basset *et al* 2003).

For instance, in human hepatoma cells T3 has been found to indirectly regulate the expression of growth hormone receptor, thus adding to the growth hormone effect on these cells. This represents

an indirect regulation of proliferation via the action of growth hormone, although the mechanisms of this indirect regulation are not understood (Mullis *et al* 1999).

The MTT incorporation assay used to determine proliferation in the present study essentially counts cell numbers via mitochondrial activity. This can often be a limitation, as in order to demonstrate true proliferation, experiments such as the Thimidine Incorporation assay, or Ki67 assay should also be completed. For example, the effect of MCT8 over-expression on the proliferation of JEG-3 cells was assessed via Thimidine Incorporation by James *et al* (2009), where they demonstrated that MCT8 did indeed reduce proliferation through such an assessment. In addition to clarifying proliferation, rejecting cell death as a cause for low cell numbers could also be a priority, to assess that cells are not dying through necrosis; using assays such as the LDH assay.

T3 Regulation of Gene Expression

TRβ1 and Cx43

Multiple factors regulate cell proliferation/cell cycle and T3 may have multiple effects on these different cellular systems. Some may be pro-proliferative and some anti-proliferative. The MTT assay assesses the sum of these effects. Cell proliferation may be regulated through the activity of TR*β*1 as indicated by studies with fibroblast cells (Porlan *et al* 2004).

TRβ1 and *Cx43* (Connexin 43) are well established TH responsive genes (Sakurai *et al* 1995; Stock *et al* 2000; Wang *et al* 2008). Cx43 is a gap junction protein involved in modulating cell–cell communication, fusion and differentiation while TRβ1 is a TR isoform and nuclear transcription factor. T3 and T4 activation of *Cx43* mRNA expression had been previously described in rat epithelial cells (Stock *et al* 1979), while *TRβ1* regulation by T3 has been investigated extensively in amphibian models such as Xenopus, as well as in various other cell types, and found to be both positive (Sakurai *et al* 1995; Wang *et al* 2008; Manchado *et al* 2009) and negative (Samuels *et al* 1989; Dace *et al* 2000; Yen 2000).

In this study, we observed a significant down-regulation of *TRβ1* with T3 treatment. Unlike *TRβ1, Cx43* was not regulated by T3 in HTR-8/SVneo. In the case of the latter, lack of Cx43 regulation by T3 is consistent with previous observations by our group in SGHPL-4 cells (unpublished data), although *TRβ1* down regulation was not observed in SGHPL-4.

These observations in Cx43 expression are not consistent with previous findings in other cell types by our group and others demonstrating induction of expression by T3 in rat epithelial cells (Stock *et al* 1979), NT2 cells (Chan *et al* 2003) and neonatal cardiomyocytes (Tribulova *et al* 2004). It would be important to confirm these changes at mRNA level and to investigate the T3 effect on these genes by assessment of protein expression.

The down regulation of *TRβ1* in HTR-8/SVneo was seen as early as 2 hours, indicating that T3 regulation of *TRβ1* may not be via genomic induction, but via pre-translational control of mRNA stability i.e. the increased mRNA degradation of TR*β1,* as demonstrated previously in GC cells by Samuels *et al* (1989). However, this would require further investigation.

In similar studies looking at gene induction by T3 in pituitary tumour cells (Kim *et al* 1998), the T3 effect on D2 mRNA expression was reported to be transcriptional, i.e. a direct effect by T3 to suppress D2 mRNA. D2, like TR*β*1 is involved in the regulation of T3 action in the cell, and like TR*β*1 was found to be suppressed as early as 2h after treatment with T3 in pituitary cells. Bianco *et al* (2002) postulated the presence of a negative TRE element in the promoter of D2, and regulation by T3 of D2 in pituitary cells was found to be direct (Kim *et al* 1998).

The majority of previous studies have reported *TRβ1* transcription to be up-regulated by THs (Sakurai *et al* 1995; Wang *et al* 2008; Manchado *et al* 2009). For example, in rat brown adipocytes, Hernandez *et al* (1996) reported increased expression of TR*β*1 (two fold increase), after treatment with T3. In addition, in rat tissues mRNA expression of *TRβ1* was described by Yen (2001), to be increased with T3 in the pituitary, yet minimally affected in non-pituitary tissues. *TRβ2* is a splice variant of TRB1 and shares a similar promoter region. *TRβ2* has been reported to be strongly negatively

regulated at the pre-translational level by T3, suggesting an effect on mRNA stability (Jones *et al* 1992). Therefore, the effect of T3 on TR*β* expression has been demonstrated to be isoform and cell type dependent.

In line with our observations (with respect to *TRβ1* down regulation), Samuels *et al* (1989) had previously reported the T3 down regulation of endogenous TRs (mRNA) including TR*β*1, in growth hormone (GH)-producing GC (rat pituitary tumour) cells. Dace *et al* (2000) more recently described the potential down-regulation of TRβ1 in GC cells, this time at the protein level. They described this to occur via the ubiquitin–proteasome degradation pathway, suggesting that T3 binding may signify rapid degradation of TR, for receptor turnover. In this scenario, they argued that the degradation of transcriptionally active TR may represent a necessary step in regulating the otherwise perpetual activation of genes by TRs which could then lead to deleterious effects on cells (Dace *et al* 2000).

TRβ1 down regulation by T3 in our cell line however, did not appear to have an overall impact on cell cycle. Our observations would suggest that TR*β*1 is not a significant regulator of HTR-8/SVneo proliferation, although this is contrary to the observations noted previously in fibroblasts (Porlan *et al* 2004); where the regulation of cell proliferation in these cells appeared to be via the action of TR*β*1 independently (independently of its hormone ligand T3) regulating

genes involved in cell cycle control. They described the role of TRβ1 in the regulation of proliferation and demonstrated that TRβ1 provoked a proliferation arrest in GO and G1 phase, lengthening the cell cycle time in the absence of hormone. They also observed that TRB1 decreased the activity of G1 cell cycle regulators including Rb and therefore the transcriptional activation of E2F-1, in the presence of T3. Thus, the repression of E2F-1 transcription was found to occur in both the presence and absence of T3 by TRβ1. This demonstrated that both T3 and its receptor TRβ1 could act independently, to elicit concomitant growth arrest (Nygard *et al* 2003; Porlan *et al* 2004).

With respect to T3 related regulation, these observations of down regulation by T3 of its own receptor TR*β*1 in HTR-8/SVneo, GC cells (Samuels *et al* 1989) and in rat pituitary cells (Yen 2001), may be indicative of an indirect regulation of other T3-mediated activities in the cell. How this down regulation occurs is presently unclear and would require further investigation in the EVT.

Deiodinases and TH Transporters

T3 does not appear to significantly regulate the expression of its own transporters MCT8 and MCT10. This is consistent with findings in other cell types, such as in SGHPL-4 (unpublished data). Presently, there is no data to support T3 regulation of these TH transporters.

Similarly, in the present study we observed no significant change in the mRNA expressions of *D2* and *D3* (which are responsible for stabilising the intracellular pool of [T3]), following T3 treatment.

There *was* however, a notable trend of down regulation of *MCT8*, *MCT10* and *D2* (responsible for activating T3); while there was a trend of up-regulation of *D3* (responsible for the deactivation of T3), in the presence of T3. Despite that only the down-regulation of *TRβ1* appeared to reach statistical significance, the effect of T3 on *D2* and *D3* expression however, was near to being statistically significant (p = 0.06).

These results indicate that the reduced D2, MCT8 and 10 mRNA expressions are a way of minimising generation and transport into the cell of T3. Similarly, a trend of increase in D3 expression is consistent with this idea of regulating T3 levels. Therefore, these data might be indicating towards T3 regulation of its own action, a form of negative feedback. In order to confirm this greater N numbers would be required, particularly as the D2 and D3 data almost reached statistically significance.

In maternal hypothyroidism and hyperthyroidism in rats and humans, the placenta does not demonstrate changes in D3 activity (Emerson *et al* 1988). Previously, groups have demonstrated no change in the expression of Deiodinase mRNA in the placenta, as a response to changes in TH status; although in rat brain and cultured human pituitary cells changes in deiodinase expressions have been observed. For the latter, *D2* mRNA is down-regulated in response to T3 treatment, while *D3* mRNA expression is down-regulated in response to reduced T3, as in hypothyroidism (Bianco *et al* 2002).

This is to be expected as Deiodinases are regulators of intracellular T3. This is also consistent with previous in vitro studies on dispersed cells prepared from human placental chorionic-decidual membranes in which up-regulation of D2 activity with T4 and T3 deprivation, and down- regulation with T4, rT3 and T3 repletion was observed (Hidal *et al* 1985). This is also consistent with observations in rat brain and cultured human pituitary cells, in which down-regulation of D2 mRNA occurs in response to T3 treatment, and down-regulation of D3 mRNA expression in hypothyroidism (Bianco *et al* 2002). The changes in D3 activity in the rat brain are relatively greater than in placenta, in response to T3 (Mori *et al* 1995). Thus, indicating that T3 effects on deiodinases are tissue- dependent.

Our group had previously assessed D2 and D3 mRNA in human primary term CTs, and in JEG-3 human choriocarcinoma cell lines (Chan *et al* 2003). Our data on primary cultures did not demonstrate any down-regulation in D2 mRNA expression with increasing T3 treatment, although increased expression of mRNA encoding D3 was observed, albeit at very high T3 concentrations (Chan *et al* 2003). There could be differences in D2 and D3 activity that are not reflected in the mRNA expression data. Therefore, the protein expressions and activities of D2 and D3 would require investigation, in future studies.

Furthermore, T3 is involved in rapid degradation of D2 mRNA by the ubiquitin pathway (Burmeister *et al* 1997; Steinsapir *et al* 2000). D3

activity may be T3 mediated rather than regulated by T3 at the mRNA level, although the mechanisms are poorly understood (Bianco *et al* 2002).

TH transporters MCT8 and MCT10 do not have TRE's in their promoters, and are therefore not expected to be directly transcriptionally regulated by TH. However, the down-regulation trends observed (although not significant) may be indicative of an indirect regulation in response to T3. The potential mechanisms that would underlie these trends of down regulation are unclear, but may reflect a cellular compensation for the increased intracellular T3 availability, by regulation of its transport. It would be important to assess the regulation of other transporters in response to T3, to see whether they are regulated. The T3 uptake and efflux in these cells would also require investigation.

Greater N numbers are required to validate whether these trends of T3 regulation reflect a real regulation by T3. Nonetheless, these regulatory trends are suggestive of a cellular drive to adjust TH concentration back to normal cellular levels as discussed previously. In this case, reducing the intracellular pool of T3 by regulating its transport, and metabolism (to rT3), will in turn regulate its activity within the cell via its receptors and other non genomic pathways.

TRs do not always mediate TH action, as demonstrated of TH action in enucleate rat erythrocyte cells and in nucleated cells that lack TR

(Galo *et al* 1981; Davis *et al* 2009). TH regulation may occur via non-genomic pathways, such as the regulation of its metabolism by deiodinases. For instance, T3 was observed to induce the degradation of D2 mRNA in the rat cerebral cortex and pituitary tumour cells (Burmeister *et al* 1997; Kim *et al* 1998; Bianco *et al* 2002; 2006).

These potential non genomic effects may be initiated at the cell membrane or cytoplasm. For example, with respect to D2, T3 mediated regulation of D2 protein expression was found to occur in the cytoplasm, via the ubiquitin proteosome degradation pathway (Steinsapir *et al* 1998; Gerben *et al* 2000).

Effect of Altering MCT8 & MCT10 Expression on Apoptosis

Apoptosis is part of normal placental development, although the regulation and exact role of apoptosis in the trophoblast is unclear. Differentiating cells in the villous compartment of placenta undergo syncytial fusion. Huppertz *et al* (2006) hypothesised that apoptosis was closely linked with this processes of syncytial fusion of CTs and the extrusion of syncytial knots from the ST. They and others had described subsets of cells to be selected for ST fusion via the asymmetric expression of various transcription factors. These cells were also found to express apoptosis-related proteins, needed for ST fusion to include effectors as well as inhibitors of the apoptosis cascade, such as Caspases or proteins of the Bcl-2 protein family respectively. Inducers of the apoptotic cascade such as FAS/CD95

and its ligand FAS/CD95L were shown to be present on EVTs from first trimester placenta. Bcl-2 expression however, has been described mostly in proliferative and post-proliferative cells (Uckan D *et al* 1997). Huppertz *et al* described a shift from the inhibition of apoptosis in the cells that express the death receptors, and the Bcl-2 family proteins (inhibitors of apoptosis) to an induction of the apoptotic 'execution program' along the invasion pathway, in which effecter Caspases (such as Caspase 3) are expressed, finally ending in cell death, deep in the placental bed (Huppertz *et al* 2006). These studies have indicated that in EVT cells, events from both the apoptosis and differentiation pathways occur.

Over-expression of MCT8/MCT10 in HTR-8/SVneo in the present study was shown to result in a significant reduction of apoptosis, while silencing MCT8 led to a significant increase in apoptosis; both effects occurring independently of T3. T3 has been reported in previous studies to reduce apoptosis in early placental EVT cells in vitro via the inhibition of Fas and Fas ligand expression and Caspase-3 and PARP cleavage (Laoag-Fernandez, *et al* 2004; Huppertz *et al* 2006). However, in this study, the effect of MCT8/MCT10 over-expression on apoptosis was observed to be T3 independent. Although this does not fit with previous observations with respect to T3 regulation of apoptosis, this observation of T3 independent effect on apoptosis are consistent with our group's previous findings in EVT cell lines and in primary term CTs (unpublished data). We demonstrated MCT8's effects in trophoblasts to be via both T3-

dependent and T3-independent mechanisms (unpublished data). In addition, MCT8 was shown by our group to adversely affect the viability of human term CTs independently of T3, an effect that was not mediated through the apoptotic pathway (unpublished data). T3 independent effects of MCT8 have also been reported previously in studies in JEG-3 and NT2s (James *et al* 2009).

The reduction of both proliferation and apoptosis in HTR-8/SVneo cells appears to be something of a paradox. However, before considering the possible mechanism of regulation of apoptosis by MCT8 and MCT10, or the same for trends in proliferation, it is important to note the correlation between the reduction in proliferation observed in these cells and the reduction in apoptosis. There is a strong possibility that the observed change in apoptosis is actually a reflection of reduced cell number.

For instance, for cells over-expressing MCT8 there was a 24% (0nM T3) and 31% decrease (10nM T3) in apoptosis, and a 36% reduction in proliferation (only significant in presence of 10nM T3). These reductions in both apoptosis and proliferation are remarkably similar. Furthermore, for cells over-expressing MCT10 there was a 12% and 19% reduction in apoptosis (in cells treated with 0 and 10nM T3 respectively) and a 29% reduction in proliferation. Therefore, given the similarity in trends, it is likely that reduction in apoptosis may have been a reflection of reduced cell number (and respective reduction in expression of Caspase 3/7) rather than a

decrease in the onset of apoptosis. In order to determine which is the true result, apoptosis should be determined in cells over-expressing MCT8 and MCT10, in an assay that normalises for cell number. Due to time constraints this was not done.

To assess the likelihood of reduced apoptosis in these cells, and whether the reduced detection of Caspases 3 and 7 is a result of reduced apoptosis we must consider what these data could be showing. Apoptosis does not represent the only way for cells to die; necrosis may also be responsible for cell death, leading to a reduction in cell number. In addition, the increased expression of early apoptotic proteins Caspase 3 and 7 may not result in cell death. Instead, cells expressing these proteins may increasingly become differentiated into ST, which would similarly not reflect apoptosis. Therefore, ruling out necrosis, the detection of other apoptotic markers, and assessment of differentiation may act to confirm whether these Caspases reflect true apoptosis induction, rather than differentiation, or reduced cell number. The alternative Nuclear-ID™ Green assay kit (Biotek) for example could be used to detect late stage apoptosis (condensed compacted chromatin).

The apparent paradox in the MTT and Caspase data may therefore be a result of reduced cell number, rather than two simultaneous pathways, where the overall result is reduced cell apoptosis and cell proliferation. However, if apoptosis is reduced following the over-expression of MCT8 and MCT10 a possible mechanism for this could

be via a possible feedback system, rather than via the action of MCT8 and MCT10 related transport of substrate.

For example, increased expression of MCT8 and MCT10 may be counteracted by increased expressions of other cytosolic proteins, or the reduced expressions of key genes in the apoptotic pathway. In order to fully investigate this hypothesis, the expressions of apoptotic marker genes could be quantified, and the activities or localisations of the respective proteins determined. Alternatively, there may be interaction between substrates of these TH transporters (MCT10 also transports amino acids) and key cytoplasmic or membrane proteins, that may play a role in cell death, or in pathways such as in the NF-KB or Ca^{2+} signalling pathway. There are many possibilities that could be explored in future studies where the expressions and activities/localisation of these proteins and markers could be explored alongside apoptosis.

This significant trend of reduced apoptosis observed in the present study is not consistent with previous observations by our group (unpublished data) in term CTs. In these cells down-regulation and over-expression of MCT8 did not cause a significant change in Caspase 3/7 activity nor HCG secretion in term primary CTs (as a marker of differentiation). In addition, previous studies in human neuronal precursor NT2 cells and MCT8-null JEG-3 cells also demonstrated no effect on apoptosis following the over-expression of MCT8 (James *et al* 2009).

These possible effects of MCT8 and MCT10, both independent and dependent on T3, on cellular processes such as proliferation and apoptosis require greater investigation to answer these numerous mechanistic questions. Prospective functional work on MCT10 and MCT8 uptake and efflux of T3/T4 will enable us to confirm whether these effects are indeed T3 mediated, or whether effects are T3 independent. In the case of the latter, other possible roles of MCT8 and MCT10 away from TH transport will require exploration.

In a recent report in murine ES cells, a role for MCT8 in promoting differentiation was indicated, where over-expression of MCT8 was found to enhance neural-lineage differentiation (Sugiura *et al*. 2007). We are not a long way from answering vital questions about the functions of MCT8 and MCT10 in the EVT, and in placenta using cell lines such as HTR-8/SVneo which have proven to be valuable tools in *in-vitro* research. Variation in cellular response can often arise between cell lines derived from the same tissue type for a number of reasons, as seen for SGH-PL4 and HTR-8/SVneo cells. While both of these cell types are derived from EVTs (SGH-PL4 cells are derived from homogenous cell isolations of first trimester EVT, whilst HTR-8/SVneo cells are derived from first trimester EVT explants (King *et al.* 2000)), there are often different processes of immortalization which may often be the cause of altered gene expressions, and changes in cell behaviour.

Despite differences in cell lines, careful interpretation of data from cell work, the use of primary cultures, and eventually animal models, will allow us to build on these foundations in understanding the roles of MCT8 and MCT10 in the EVT and in the placenta.

Conclusions

MCT8 and MCT10 appear to be implicated in the regulation of trophoblast proliferation and apoptosis via both T3 mediated and T3 independent pathways. In HTR-8/SVneo, T3 on its own does not alter proliferation, nor does it significantly regulate the expressions of the various genes involved in its metabolism. However, it does regulate the expression of its receptor TRβ1, suggesting a form of compensation to the changed T3 status of the cell.

Findings that alterations in MCT8 expression can alter the behaviour of various cell types, including EVT, neuronal and choriocarcinoma cell lines, in the absence of T3 suggest that MCT8 may have T3 independent effects. Further research is required to identify any alternative substrates for MCT8 transport, which may be implicated in the regulation of trophoblast functions such as apoptosis and differentiation. The data from this study also suggest that MCT10 may be implicated in the regulation of these processes via T3 independent effects. Perhaps this is less surprisingly the case, as MCT10 is known to transport other substrates as well as transporting TH; specifically aromatic amino acids such as tryptophan. Thus, there is a possibility that MCT10 may be involved

in the regulation of trophoblast function via amino acid mediated pathways or through its ability to transport alternative substrates which may be implicated in cell cycling and metabolism.

The characterisation of the effects of T3 on HTR-8/SVneo thus far has offered insight into the possible roles of these proteins in EVTs and in placental development. Future work in placental primary cells (first trimester EVTs), will help to validate and support these findings.

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